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# PHYSIOLOGICAL REVIEWS

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## BIOLOGICAL RHYTHMS AND CYCLES

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CHICAGO, ILLINOIS

RHYTHM, CYCLE AND PERIOD are terms variously and often interchangeably applied to courses, rounds or series of repetitive or recurring phenomena or events, conditions or states, of inorganic or organic nature, the fluctuations sometimes being designated as waves, tides, swings or pendulations. Regularity of succession, both with respect to duration of the period and order of occurrence of its constituent phases, is emphasized in some definitions of these terms, but many cycles recur at irregular intervals of time.

For the purposes of this review, the term *rhythm* will be used to designate a regularly recurring quantitative change in some particular variable biological process, irrespective of whether or not it takes place in a cell, tissue, structure, organism or population. Two conditions are necessary to make such a recurring change into a rhythm: *a*) it must be extrinsic in origin, depending upon a regular change in the environment, such as light or temperature, usually associated with terrestrial or cosmic periodicity, developing in each biological system *de novo*; and *b*) when fully established, it must persist for some time, even when the environmental changes are absent. Except that the regulating mechanism need not be nervous, a rhythm may be likened to a conditioned response, which is also individually acquired and depends on an extrinsic reinforcement for its establishment, yet will persist for a shorter or longer period of time in the absence of such reinforcement. This is the sense in which Welsh (1) used the term in his review of diurnal rhythms. The term *cycle*, on the other hand, will apply to repetitive series of events or to successive changes of state, thus being either qualitative or quantitative in nature, and its one distinctive feature is the order of occurrence, rather than duration. Cycles are intrinsic in origin and have to run their course to be completed. They may be influenced by internal and external conditions, which may affect them quantitatively, but seldom qualitatively. A good example is the cardiac cycle which may be affected, in duration and magnitude of change, by drugs, nerve impulses, temperature variations and so on, but which is intrinsic in origin and development. A third type of biological recurrence, which, like the rhythm, is extrinsic in origin, but is so directly dependent on environmental changes that it shows no persistence of variations when the external conditions are made uniform, will be referred to as causal, synchronous, associated or coupled *periodicity*. The regularity or irregularity of periodicity is a function of the external

variation with which it is coupled. Diurnal periodicity may be quite regular, while meteorological periodicity is not.

Observations of, and speculations on, terrestrial and cosmic periodicity, and its effects on the living, extend back into antiquity. Omens and portents of things to come, as developed by astrologers, are still printed in the metropolitan newspapers of today. Aside from attempts by individual philosophers and scientists to make a rational analysis of the relations between the non-living and living worlds, a collective attack on the problem was made by the organization of the International Society for the Study of Biological Rhythms, which held two conferences: the first at Ronneby, Sweden, in August 1937, and the second at Utrecht, Netherlands, on the eve of the outbreak of World War II, in August 1939. The Proceedings of these conferences (2) contain discussions of a variety of periodicities in plants and animals. Schultz, a participant in the first conference, in treating 'Lebensrhythms und Psychoterapie' lists six categories or polarities in which life-rhythms can show changes: *a*) tempo, as acceleration and slowing, *b*) amplitude, as excitation and inhibition, *c*) intensity, as augmentation and diminution, *d*) quality, as enlivenment and blunting, *e*) modulation, as mobility and rigidity, and *f*) 'Duktus,' as tension and relaxation.

On the factual side, there are a number of review articles describing and analyzing the accumulated data on many rhythms, cycles and periodicities, largely, however, diurnal ones. Freeman and Hovland (3) reviewed papers on the diurnal variation in human work output, energy expenditure, muscular activity, higher mental processes, as well as factors which tend to affect the diurnal curves. Jores (4, 5, 2) also devoted his several reviews mainly to the human diurnal rhythm. In one (4), the rhythms taken up included sleep and associated phenomena, heart rate, blood pressure, respiration, body temperature, oxygen consumption and carbon dioxide production, blood and its constituents (plasma, erythrocytes, their sedimentation rate, hemoglobin, leucocytes, thrombocytes), water loss, excretion of solids, visceral activities (liver, stomach) and endocrines (adrenals and hypophysis). This was followed up with an inquiry into the endogenous and exogenous origins of the rhythmicities, as well as the rôle of rhythms in pathology. Another review (5) covered 24-hour periodicities in plants (opening and closing of flowers, pollen production, nectar and sugar-content, movement of leaves, electrical excitability of mimosa, etc.); animals (pigment movement in Salamander larvae, pollen-gathering by bees, etc.); and in man, diurnal variations in plasma chemistry (proteins, sugar, tryptophane, urea, uric acid, sulphates, chlorides, bilirubin), electrical skin resistance, sensitivity of teeth to painful stimulation and incidence of births and deaths.

Welsh (1) reviewed papers on diurnal variation in light production, movements of rods and cones, retinal pigment, surface color, general activity, metabolic rate and body temperature in a number of invertebrates and vertebrates, including birds and mammals. Herring and Brody (6) treated, in addition to some of the above-mentioned functions, the diurnal variation in milk production by cows, and metabolic activity of pigeons.

Five chapters in *Sleep and Wakefulness* (7) dealt with periodicity. Into them were incorporated brief reviews of papers on the establishment, maintenance and modification of the diurnal body temperature rhythm, diurnal variations in somatic and visceral activity, as well as meteorological and seasonal influences. Bissonnette (8) reviewed the gradually accumulating evidence that seasonal variation in the duration of daylight is the important factor in the establishment of animal sexual periodicity in some birds and mammals. Park (9) analyzed the problem of nocturnalism—the adjustment of many species to predominant activity during the night. In addition to a theoretical discussion of the general problem, Park collected a bibliography of over 300 titles. Petersen (10) collected data on meteorological and cosmic influences on physiological and pathological processes in the human individual and in populations, the latter from vital statistics tables.

This 'review of reviews,' though by no means exhaustive, is intended to guide the reader in looking for papers on periodicity, as references to the literature given

in them will not be relisted here, by and large, but some previously reviewed papers may be cited, in support or refutation of more recent findings.

#### PERIODS OF LESS THAN 24 HOURS

Omitting the brain-potential, heart and respiration cycles, as they have been the subject of many reviews, one finds an activity-rest cycle which in some animals is measured in minutes or hours. Szymanski (11), a pioneer in this field, introduced the term 'polyphasic' to designate animals showing several periods of activity in 24 hours, as contrasted to 'monophasic,' animals with only one such period. Although I have contributed as much as anyone to the general acceptance of Szymanski's nomenclature, I suggest that the terms be discarded, as each period comprises a rest phase as well as an activity one. More appropriate terms would be polycyclic and diurnally rhythmic or periodic, depending upon whether or not the single alternation per 24 hours persists under uniform environmental conditions. Szymanski found 10 activity-rest cycles per 24 hours in the white rat, 16 in the white mouse, 19 in the gray mouse, and 16 to 21 in the rabbit. Richter (12) definitely linked the 2-hour rat activity cycle with a gastric motility, hunger and feeding cycle. Davis (13) observed a similar 2 to 4-hour feeding cycle in another rodent (the short-tailed vole), and Stier (14) found that 40-day-old mice alternated 25 to 135 minutes of activity with 15 to 160 minutes of quiescence. Richter (12) also detected 2- to 4-hour cycles of drinking, urination and defecation.

The human infant, typically polycyclic, to use the revised nomenclature, shows 5 to 6 activity cycles in 24 hours, according to Szymanski (15). In a study of motor activity of 4 infants, from the second to the ninth day after birth, on a self-demand feeding schedule, Marquis (16) obtained figures not much different from Szymanski's: 5 to 10, with an average of 7.3, per 24 hours. Although the average length of this cycle was 3.2 hours, about 20 per cent had a duration of less than two, and a little over 10 per cent, more than five, hours. It is interesting to note that 38 infants on a fixed 3-hour feeding schedule, studied simultaneously, showed an almost uniformly low activity during the hour preceding the feeding, whereas 18 infants on a 4-hour feeding schedule, while similarly inactive during the third hour, were very restive during the fourth. The infant's cycle then is, like the rat's, an expression of recurring hunger.

What appears to be an intrinsic cycle of irritability was described in the amphibian *Amblystoma punctatum* by Coghill and Watkins (17). Thirty specimens, tested concurrently, at 15-minute intervals, during their development, showed a pronounced synchrony in the fluctuation of their sensitivity to tactile stimuli. Periods of marked lowering of threshold occurred at intervals of 8 to 10 hours during 64 hours of observation. Hunger was excluded, as every cell in the body of the larva had its own food in the form of yolk. The authors concluded that fluctuations in irritability must have their origin in "an intrinsic periodicity of organismic processes," which "may be the primary factor in the activities and sleep of animals generally."

A variety of rhythms and periodicities have been linked with the ebb and flow of ocean tides that have a period of about 12 hours. Hoffmann (18) mentions the marine invertebrate, *Actinia equina*, which retracts its tentacles and closes its sphincter during ebb-tide for several days after it had been removed to a laboratory



tank. In German communities on the North Sea coast there is a popular notion that births occur during high tide, not only of human beings but also of livestock. A farmer will not stay up at night to assist a cow in labor if the tide is low. Similar reports have come from Japan. Kirchhoff and Harfst (19) studied the incidence of 1360 births in coastal towns and found a distinct, though not overwhelming, preponderance of births during flow-tide.

In the three-hour period straddling the height of the tide there were 378 births, as against 285 during a similar period at ebb-tide, or a ratio of 4 to 3. They could not, however, confirm the equally prevalent notion that labor pains are strongest around flow-tide. Also, in an analysis of 7074 births at Kiel, on the Baltic, where there are practically no tides, the births were found to be uniformly distributed over the hours. What the cause of this tidal periodicity may be is not known.

#### DIURNAL PERIODS

Most of the publications pertaining to rhythmicity and periodicity deal with the 24-hour, or diurnal, period of day and night. For purposes of classification, the papers may be divided according to the phenomenon studied or the type of animal involved. For the former, there are reports on somatic activity or motility, visceral activity, and body temperature; for the latter, water-inhabiting invertebrates, air-inhabiting invertebrates, poikilothermal vertebrates, birds, mammals and man. Diurnal rhythmicity can be distinguished from mere periodicity by the criterion of persistence, following the suggestion of Welsh (1).

*Activity in Invertebrates.* Copepods swim nearer to the ocean surfaces at night than in the daytime. Welsh, Chace and Nunnemacher (20) hold light (or variations in light intensity) to be the most important external factor in this diurnal migration. That no true diurnal rhythm is developed in these animals was shown by Schallek (21) who observed the up-and-down migrations of the copepod *Acartia tonsa*, kept in tall glass cylinders, in the laboratory, with the alternation of day and night, and by artificially regulating the illumination. "Migration ceases under constant conditions; it can be reversed by illuminating the animals at night and keeping them in the dark by day; and the normal 24-hour behavior can be compressed into four hours."

Although the crayfish exhibits a diurnal activity rhythm under natural conditions (greater activity at night), the cave crayfish, *Cambarus pellucidus*, obtained from Mammoth Cave, and made to record its motor activity by a modification of the Spencer ichthyometer, was found by Park, Roberts and Harris (22) to be entirely arrhythmic, as far as day and night were concerned. However, this animal showed a photonegative orientation.

Park and Keller (23) captured a variety of nocturnal insects. The forest cockroach, whose peak of activity is naturally between 8 p.m. and midnight, could be kept in continuous motility when placed in a dark room, but, under the same conditions, the forest mycetocole preserved its night activity and daytime rest. Likewise, Park (24) found the black passalid beetle to be arrhythmic in the dark, while millipedes persisted in their night activity after 10 to 18 days in total darkness. Crawford (25), whose review of habits and characteristics of nocturnal animals has an extensive

bibliography on the subject, is puzzled by the factors that make an animal active during one or another portion of the 24-hour period: "The fact that the long-horn grasshoppers are in some species nocturnal, in some diurnal, while practically all are apparently protectively colored and all face the same conditions, is difficult to explain. Along with almost any form, active at night for any apparent reason, can be found a closely related form not active at night, yet exposed to the same conditions."

For several species of cockroaches and the mosquito *Anopheles maculipennis*, the immediate cause of nocturnal activity was determined by Necheles (26). He attributed the hiding of these insects during the daytime to the high temperature and low humidity of the air which could cause excessive loss of water by the animals. Mellanby (27) studied the activity of both cockroaches, *Blatta orientalis*, and bed-bugs, *Cimex lectularius*, in a natural infestation of a laboratory rat-room. Activity was determined by the number of specimens caught in a trap, which was emptied at 3-hour intervals. For the bed-bugs, at least, Mellanby could find no correlation between activity and either temperature or humidity. In the cockroaches, artificial illumination almost entirely abolished activity (28), but in bed-bugs it only reduced it 65 to 80 per cent. Cockroaches almost never appeared during the day and were caught in greatest numbers prior to midnight, while bed-bugs were out in small numbers at all times and had their greatest activity after midnight. Only cockroaches had a diurnal periodicity, the bed-bugs really operating on a 6-day cycle, but, being photophobic, came out mainly at night. According to Mellanby, in the cockroach "rhythmic activity is apparently controlled by an internal mechanism."

Kalmus (29) described and analyzed the "hatching rhythm" of cultures of *Drosophila* flies. The larvae naturally emerged during the hours preceding sunrise, whether kept in colonies or isolated from each other. Light was the controlling factor, an inverted illumination schedule resulting in an inverted emergence rhythm. In cultures that hatched in the dark, the emergence was aperiodic, but the 24-hour rhythm persisted when the cultures, previously illuminated diurnally, were later kept in constant darkness. Acquisition of the diurnal rhythm seemed to be entirely "athermochronous," i.e., with the usual 24-hour alternation of light and darkness; temperature variations from 12 to 28° C. had no effect on the frequency of emergence. However, in cultures, which developed the 24-hour rhythm at very low or high environmental temperature, subsequent continuous darkness led to a slight lengthening (up to 26 hours) or shortening (down to 20 hours), respectively, of this previously acquired diurnal rhythm. Furthermore, when the change in temperature was induced *simultaneously* with the initiation of darkness, the emergence rhythm could be brought down to 16 hours (by warmth) or raised to 60 hours (by cold). Diminishing the oxygen tension in the culture bottles had the same effect as lowering the temperature. Kalmus was able to establish the 24-hour emergence rhythm by a single period of illumination lasting 2 to 4 hours. Here, too, the effect of temperature depended upon whether or not the change was made before or after the light was turned off. If the temperature was altered at the beginning of the lighting period and then held constant, a regular 24-hour rhythm developed in a previously aperiodic, dark-kept culture. If the temperature was changed immediately after turning off the light, the rhythm was altered. The exposure to light, thus, had the effect of delaying emergence

by about 20 hours and, at the same time, causing the appearance of a 24-hour rhythm of emergence in constant darkness, provided that the temperature was not changed as soon as darkness began.

*Color Changes in Invertebrates.* Numerous instances of diurnal rhythms in the eyes and integument of invertebrates can be found in the reviews of Welsh (1) and Brown (30). In the Bermudan isopod, *Ligia baudiniana*, which is dark during the day and light-colored at night, Kleinholz (31) demonstrated that the diurnal alternations of pigment-cell dispersion (darkness) and contraction (lightness) were not due to a rhythm of exhaustion and elaboration of hormone production by the sinus-gland: injection of aqueous extracts of heads of *Ligia*, either in the dark or light-pigment phase, into the body spaces of dark *Ligia* always brought about a lightening of the color. Brown (30) does not rule out a hormonal diurnal rhythm in some other structure, not located in the head.

I was not only able to confirm Kleinholz's finding that the diurnal pigment rhythm of *Ligia* persisted in the dark, but developed an artificial 18-hour rhythm or periodicity, by subjecting the animals to a succession of periods of 10 hours of light and 8 hours of darkness (32). Paradoxically, in the newly-induced rhythm, the *Ligia* were light, instead of dark, during the illumination phase of the 18-hour period.

*Activity in Poikilothermal Vertebrates.* There are very few reports on diurnal variation in activity of fishes. Spencer (33) graphically recorded the movements of four species of fresh-water fishes, kept in a laboratory basement in the summer, with little diurnal variation in water temperature. The carp and mud minnow were swimming mostly at night, while the sunfish confined its activity almost entirely to daytime hours, and the rock bass showed little diurnal variation. Whether the diurnal changes in activity were true rhythms, or a periodicity following the alternation of light and darkness, was not stated.

A peculiar inversion of the diurnal periodicity in migrating salmon seems to be connected with their age or the direction of water-flow. According to Neave (34), the upstream migration of salmon takes place mainly during daylight hours. Light, rather than volume or temperature of the water, appeared to be the determining factor. Salmon fry, on the other hand, are reported by Pritchard (35) to swim downstream only at night. Even moonlight has a slowing effect on their migration.

Kalmus (29), unlike Coghill and Watkins, did not observe any cycle of less than 24-hours' duration in *Amblystoma* larvae. He did find the latter active at night and quiescent in the daytime. He showed this alternation to be a diurnal rhythm by its persistence in continuous darkness and by its reversibility under an inverted 24-hour schedule of illumination. After hypophysectomy the diurnal rhythm no longer persisted in the dark, but thyroidectomy was without effect.

*Color Change in Vertebrates.* Welsh (1) reviewed reports of diurnal skin pigment dispersion rhythms in the lamprey and salamander. The rhythms persisted in darkness, but were abolished by continuous light. Rahn and Rosendale (36) obtained similar results with the lizard, *Anolis carolinensis*, which is normally bright green at night and dark brown in the daytime. The color rhythm persisted for as long as 18 days in continuous darkness, but uninterrupted light abolished it in some

animals. Hypophysectomy destroyed the rhythms, the lizards becoming permanently green, due to pigment concentration. The authors concluded that the rhythm they studied was really one of melanophore hormone secretion by the pars intermedia of the hypophysis, resulting from intermittancy of light.

*Activity in Birds and Mammals.* Cook and Fredrickson (37) made direct observations of the frequency of movement of canaries for one hour, five times daily, at 9 A.M., noon, 3, 6, and 9 P.M. The animals were exposed to one of two light intensities, a low one, just strong enough to make the birds visible, and another, eight times the intensity of the lower one. Aside from being more active under the influence of the stronger light, the canaries showed a distinct diurnal curve of activity with either light, the highest average number of groups of movements per minute (up to 2.67), and shortest interval between groups occurring at 3 P.M. The curve was not quite symmetrical, as activity was smaller at 9 P.M. than at 9 A.M.

Among mammals, rodents appear to be the animals of choice for the study of diurnal variation in activity. Captured wild deer mice confine their activity to night hours, according to Johnson (38). Davis (13) found that the vole has, in addition to the 2- to 4-hour feeding cycle, a diurnal rhythm of activity, with a maximum at night. The wood rat sleeps in the daytime, but is quite active during the night (39). When not hibernating, the hedgehog is active for about 6 hours out of every 24, but the activity is limited to night hours. Herter (40) described three nocturnal activity periods in this animal: a maximal one between 6 and 8:30 P.M., and two others, less intense, at 12:30 to 2:30 A.M. and 4:00 to 5:30 A.M. The chipmunk, *Tamias striatus griseus*, on the other hand, kept in constant darkness, with uniform air temperature and relative humidity for 44 days, was found by Park (41) to run up 82 per cent of its activity between the hours of 7 A.M. and 6 P.M.

The amount and distribution of daily activity are markedly affected by partial or complete deprivation of food. Richter's rats, when starved but supplied with water, increased their activity for 2 to 3 days, then gradually descended to almost complete inactivity on the eighth day (42). Shirley (43) could modify the distribution of activity in rats, under continuous light, by feeding them at different hours. When fed at 1:30 P.M., the animals remained inactive till evening, were moderately active between 6 P.M. and 1 A.M., then, after almost complete quiescence for the remainder of the night, had an outburst of activity during the forenoon. A maximum was reached in the hour just before feeding, which accounted for 21.8 per cent of the day's total activity. Somewhat the same distortion of the diurnal pattern of activity can be seen in the 'actograms' of mice, published by Achelis and Nothdurft (44). On a qualitatively and quantitatively adequate diet, placed in the cages between 11 A.M. and noon, their mice had a diurnal activity rhythm, with considerable movement between 6 and 11 P.M. and smaller periods of activity distributed irregularly over the remainder of the 24 hours. When the diet, while qualitatively still adequate, was so decreased that the mice at first lost some weight and then were just able to maintain it at a lower level, the actogram was completely changed: feeding was followed by complete inactivity which lasted till morning, then a greatly increased activity during the forenoon (pre-feeding) hours. The authors suggest that the actograms be used as nutritional indices. Results, in line with the above

on rats and mice, were obtained by Engelmann and me (45) on rabbits, which, with food in the cages at all times, were more active at night than in the daytime, the minimum occurring around noontime. When food was available only between 9 or 10 A.M. and 3 or 4 P.M., the animals ate about 25 per cent less than previously, but the incidence of activity changed sharply, amounting almost to a reversal of the pattern, with a greater diurnal range. The rabbits were most active during the day, increasing their movement not only in the hours just prior to feeding, but also after food was supplied. Restricting the period of availability, and thus indirectly the quantity, of food rendered the rabbits more definitely active and diurnally periodic.

Wald and Jackson (46) echoed the conclusions of Achelis and Nothdurft, on the basis of global daily activity of rats, as determined by counting the revolutions of running wheels, to which the animals had access. Starvation increased the daily run four- or five-fold. Their conclusion: "High running is not, therefore, a reliable sign of well being and optimal performance. It may be a sign of want. When healthy, intact animals are most completely provided with their needs they run minimally. This relation may be used as a criterion of dietary adequacy." The hyperactivity connected with the need of satisfying some bodily want corresponds to the "wakefulness of necessity," postulated in the evolutionary theory of sleep and wakefulness (7).

The sheep and pig show entirely different diurnal variations in activity. Curtis (47), by attaching pedometers to his animals, found both to be predominately active during the day, the pig a little more than the sheep. The percentage of the 24-hour activity that occurs between the hours of 6 A.M. and 6 P.M. is 78 for the sheep and 88 for the pig. The guinea-pig, according to Nicholls (48), is practically continuously active, with no diurnal variation whatsoever. The effect of constant light was to decrease its activity by 4.6 per cent, as compared to the figures obtained under constant darkness. It is undoubtedly an exaggeration to state, as Nicholls did, that the guinea-pig is active 89 to 93 per cent of the time, since no measurements of the actual duration of activity were made. In our study of the diurnal distribution of activity of rabbits (45), movements of the animals activated an electric clock, the second hand of which closed an electric circuit once every sweep. In this way each minute of activity, whether continuous or made up of the sum of several shorter periods, was recorded on a kymograph by means of a signal magnet. The rabbits, which seemed to be moving all the time, spent as little as 4 minutes, and seldom more than 20 minutes, in actual motility, out of each hour—an average of  $5\frac{1}{2}$  to 6 hours of activity out of every 24.

The majority of the experimental procedures in the study of the diurnal activity rhythm involved changing the natural alternation of light and darkness, in order to extinguish, reverse or modify the rhythm. Slonaker (49) was able to decrease the night activity of the rat by continuous illumination. Richter (42) showed that this diurnal rhythm persisted under conditions of constant darkness. Browman (50), using both continuous darkness and continuous light, obtained different effects on the diurnal activity rhythm of rats. In the dark, the rats retained their usual rhythm. Light, kept on for 4 to 9 weeks, caused a peculiar disorganization of the rhythm: a repeated back-and-forth shifting of the ~~maximum~~ activity from

solar night to solar day hours, each phase lasting 5 to 6 days. Johnson (38) in the wild mice, Davis (13) in the vole, Wolf (51) in the dancing mouse, and Griffin and Welsh (52) in the bat, all found that constant darkness did not abolish a previously established diurnal activity rhythm. In mice, reared in darkness from birth, there did not develop a diurnal activity rhythm (51). Hunt and Schlosberg (53) blinded or castrated rats, but these operations did not extinguish the activity rhythm, although the diurnal fluctuations tended to be less marked.

Browman (54, 55) compared the effect of alternating light and darkness and high and low room temperatures on the activity of normal and blinded rats. Light inhibited the activity of normal rats, regardless of temperature variations. In the blinded rats (as well as in normal rats kept in the dark) a diurnal activity periodicity could be established by changing the room temperature every 12 hours. The animals were more active during periods when the temperature was 16 than when it was 27° C., whether the alternations did or did not coincide with solar day and night. In normal animals, the total activity was substantially unchanged by turning the light on and off every 12 hours or every 8 hours.

Reversal of the activity rhythm by an inverted daily schedule of artificial illumination and darkness was achieved by Johnson (38), Browman (50, 54, 55), and Hunt and Schlosberg (53). However, attempts to establish 16-hour activity rhythms, by Johnson (38) in wild mice, and by Hemmingsen and Krarup (56) in rats, resulted in failure, though light usually had a depressing effect on activity.

Contrary to data reported on poikilothermal vertebrates (29, 36), Levinson, Welsh and Abramowitz (57) could not abolish the diurnal activity rhythm of the rat by hypophysectomy. The operation itself depressed activity, the latter never reaching the preoperative level. The diurnal rhythm was maintained in the dark and could be reversed by changing the schedule of illumination.

*Activity and Performance in Man.* Human beings are discussed separately from other mammals, because in the former, social influences are much more important than changes in the physical environment in the establishment and maintenance of many diurnal rhythms. It is true that customs, with respect to daily schedules of sleep, work, meals and play, represent a group adaptation to the 24-hour alternations of light and darkness, but for the individual, it is more a matter of acculturation than acclimatization. The activity of newborn babies is governed in the main by the gastric hunger cycle. However, variations in room temperature and in the amount of noise make themselves felt in the early days of the baby's extrauterine life. In the four neonates on a self-demand feeding schedule studied by Marquis (16), the intervals between 'demands' were, on the average, 2.86 hours by day and 3.61 hours at night, the day-night difference being significant for each baby. Periods of quiet exceeding 5 hours predominated at night (23 out of 28), whereas periods of less than 2 hours' inactivity occurred mainly in the daytime (34 out of 48). In Gesell's book (58) there is a report of observations made on the activity and condition of a healthy infant (*J.*), at 30-second intervals, from 20 minutes after birth till the age of 15 days. Not only was there a gradual diminution of the number of sleep periods, and therefore an increase in the duration of individual naps ('consolidation' vs. 'segmentation'), but the sleep-wakefulness ratio soon showed a diurnal

periodicity. During the third day the baby was awake 40 per cent of the time, with no diurnal variation. By the sixth day, the percentage of wakefulness increased to 48 between 6 A.M. and 6 P.M., but fell to 35 during the 12 night hours. Further progress consisted of a slight increase in daytime wakefulness, but a marked decline in number and duration of waking periods at night.

Mullin (59) followed the amount and diurnal pattern of activity of a baby during the first 15 months of his life. Activity was greater as time went on, but the day-to-night ratio, for the first two months close to 1, was raised to 2 in the third and fourth months, until by the seventh month it exceeded 3, not only through an increase in activity during the day, but also a decrease during the night, as compared to the earlier months.

In older children and adults, the diurnal pattern of activity is influenced by a number of social factors, compulsory or elective character of the activity (work or play), financial or prestige incentives, and the personality or temperament of the individual. Speed and accuracy in carrying out a short-lasting task, under laboratory conditions, usually reveals a diurnal variation, but curves of performance seem to vary with the experimenter, his subjects, and the activity studied. Freeman and Hovland, in their review (3), prepared a table of data on diurnal variation in sensory, motor and mental performance, revealing four types of performance curves: a continuous rise through the whole period of observation, a continuous fall, a rise followed by a fall, and a fall followed by rise. While there is no present explanation for the last type of curve, the first three can easily be correlated with the peculiarity of individual adaptation to the diurnal routine of existence, as will be shown in the section on body temperature.

In our experience (7) the third type performance curve, a rise followed by fall, was prevalent in a variety of muscular and mental tasks. Among them were: dealing and sorting playing cards, mirror drawing, copying texts, transcribing codes, multiplying eight-digit numbers, color-naming, hand-steadiness, reaction time, incidence of 'blocking', etc. The activity of the extrinsic eye musculature is of special interest, as binocular eye fixations go on throughout the waking period or as long as the eyes are open, irrespective of whether or not the individual is at work, play or rest. Schreider and I (60) have determined the variation in the ability of several subjects, kept awake overnight, to fixate their gaze on a point for five-minute periods, at 2-hour intervals. An ocular imbalance leading to diplopia became more marked with time, reaching its greatest extent in the early hours of the morning, when sleepiness was extreme. But later in the day, as the subjects became more wide-awake, good binocular fixation was gradually restored. The fatigue of continuous use of the eye-muscles was, thus, aggravated or attenuated in the different phases of the diurnal rhythm.

*Body Temperature of Mammals and Birds.* There is little to add to the older observations of Galbraith and Simpson (61, 62). The rabbit, they found, had its highest rectal temperature in the evening. This was confirmed by us (45), but was shown to be linked to the feeding habits of this species. When we restricted the food intake to a few hours (9-10 A.M. to 3-4 P.M.), the rabbits' temperature showed a marked rise during that period, reaching a maximum in the afternoon, instead of in the evening.

In the monkey, Galbraith and Simpson (61) were able to reverse, as well as to extinguish the rather marked diurnal temperature curve. Gradual extinction was accomplished in constant darkness in one week.

The same authors (62) reported the following characteristics of the diurnal temperature variations in birds: *a*) dependence on activity habits—the owl, for instance, having its highest temperature at or after midnight; *b*) higher temperatures in females than in males; and *c*) a greater diurnal range the smaller the bird,—the value for the duck being  $0.92^{\circ}\text{C}.$ , and for the thrush  $4.27^{\circ}\text{C}.$  Hildén and Stenbaek (63) succeeded in reversing the diurnal body temperature of birds by inverting the alternation of light and darkness.

*Body Temperature of Man.* It has been amply demonstrated by Jundell (64) and others (59, 65) that the diurnal body temperature rhythm in man is developed by acculturation and maintained by and through adherence to the socially prevalent routine of living. It remained to be demonstrated that the temperature level and the shape of the diurnal body temperature curve were related to, and could be used as an index of, efficiency, as measured by the speed and accuracy of performance (7, 66). Furthermore, while under usual conditions of civilized existence, the body temperature reaches its peak during the wakefulness phase of the 24-hour period, and its minimum during sleep, there are distinct individual variations in mean composite diurnal temperature curves (7, 67). So-called morning types reach their maximum temperature early in the day. Their performance is better immediately upon getting up in the morning than just prior to going to bed at night. This may seem logical, since sleep is universally considered as having a restorative effect, but the body temperature in morning persons is higher in the morning than at night. At either end of the waking period, however, temperatures are rather low and performance poor. If a morning individual is first tested in the laboratory two or three hours after he gets out of bed, he may by that time have reached his body temperature peak and his performance will show a downward trend, falling into the second category of Freeman and Hovland's diurnal curves. Conversely, an evening type, who 'comes to life' toward the end of the waking period, dislikes going to bed at the conventional retiring hours and 'hates to get up in the morning', will show a rising body temperature curve during the day, and his performance will fit into the first group of curves. The in-between individual, whose body temperature rises till the middle of the afternoon and then begins to fall, will show an increase in efficiency, followed by a gradual decrease. There are many gradations between the two extremes, but recently an entirely different temperature curve has been found (67) where, after an early morning rise, there is a temperature plateau for the rest of the day, and sometimes well into the evening. Such plateaus were obtained in two factory foremen, and it is impossible to say how prevalent they are among gainfully employed persons who have to do a daily stint of work at a uniform rate for several hours.

The frequency distribution of body temperature figures for each hour, on many successive days, is another personal characteristic. In some individuals, the body temperature variations for any hour, over a period of several months, may exceed the mean diurnal body temperature range (67); in others the repetitiveness of successive diurnal curves is very close. In almost all persons, when a large number of readings



have been made, the frequency distribution histograms of temperature for any given hour of the day and night will form a symmetrical probability curve.

The human diurnal body temperature curve can be shifted or completely reversed by changes in the daily routine (7, 67). Burckard and Kayser (68) reversed the body temperature curve of a 27-year-old bedridden idiot, by changing the hours of illumination and feeding.

Attempts to establish body temperature rhythms of more or less than 24 hours by a changed routine of living led to success in some subjects and failure in others (7). The length of the artificial period is of importance. Adjustment could be made to 21- and 28-hour periods (8- and 6-day weeks), but not to 48 or 12 hours (7, 68).

Magnussen (69, 70) advanced the view that "there is a close connection between peripheral vasodilation and vegetative preparedness for sleep", as shown by "rhythmical diurnal variations in skin temperature of man." Actually he studied only the temperature of the skin of the feet (toes and dorsal surfaces), and found that in this region the skin temperature might suddenly go up as much as  $10^{\circ}$  C. an hour or two before the onset of sleep. But foot or toe skin temperatures are not even indicative of temperatures in general. We observed at times opposite variations in the skin temperatures of the hands and feet (71). Following a meal there is usually a rise of both finger and toe skin temperature, but muscular relaxation leads to a drop in finger temperature and a simultaneous rise in toe temperature. In the pre-sleep hours of the evening, there is thus a combination of two toe-skin temperature-raising influences (meal and muscular relaxation), and they, rather than a diurnal variation in general sympathetic activity, are responsible for the elevation of the skin temperature of the feet.

*Other Diurnal Variations in Animals.* The diurnal glycemie variation in rats, with values about 10 per cent higher at midnight than at noon, was definitely related by Pitts (72) to the animals' feeding habits. Rats trained to feed during the day showed a reversal of the blood-sugar curve, even though they were more active at night. The diurnal variation disappeared after the animals were deprived of food for 36 to 48 hours. A similar coupling with feeding habits was earlier reported by Higgins, Berkson and Flock (73), for gross weight of the liver (as well as its water, glycogen, protein and fat content) in rats.

Levinson (74) determined the variation in intermedin content of the rat's blood, at 3-hour intervals, through 24 hours. The assay was based on the degree of melanophore expansion produced in pale frogs. More intermedin was found in the blood at night, when the rats were active, than in the daytime. In this connection, it may be mentioned that hypophysectomy, according to Jores (75), does not entirely abolish the diurnal variation in glycogen and bile pigment content of the rat's liver, which is in line with the effect of this operation on its diurnal variation in activity (57).

Swan (76) reported a diurnal fluctuation in blood lactate in the normal resting dog. There were two peaks, one at noon and another at midnight, the latter lacking the well-sustained character of the mid-day rise.

In the normal daily 16-MU increment of dentin on the incisor of the rat, Schour and Steadman (77) demonstrated a succession of dark and light layers. The former, well calcified, was laid down in the daytime, the latter, poorly calcified, at night.

These stratifications are probably a reflection of the well-demonstrated feeding habits of the rat (72, 73).

A different aspect of growth was studied by Carleton (78)—mitotic activity in the epidermis and epithelial cells of hair follicles of new-born mice, in the main, but also in a few recently weaned rats. The animals were killed at 4-hour intervals and the number of cells with mitosis out of a 5000-cell count recorded. The highest rate of cell division occurred at 8 P.M., lowest at noon. Attempts to shift the incidence of maxima and minima in the mice by artificial illumination for 12 hours were unsuccessful. The rhythm was still present when animals (both mice and rats) were kept in the dark for 7 to 14 days, but was destroyed by exposure to continuous light.

*Other Diurnal Variations in Man.* Although the heart rate changes continually, mean values for successive hours of the diurnal period yield a definite curve which is, by and large, parallel to the body temperature curve (67), except for the frequent postprandial increase. The latter may cause a rise in heart rate after the evening meal, when the body temperature is falling rapidly. By reversing the routine of living, one can invert the diurnal heart rate curve, along with the inversion of the body temperature rhythm. The heart rate variation probably represents a complex periodicity, depending directly upon the body temperature, a change of 1°F. causing a swing of 10 to 20 heart beats per minute.

Diurnal variation in blood sugar is bound up with food intake, as it disappears on fasting, according to Sweeney (79). Shaw (80) found two waves in the diurnal variation in leukocytes (granulocytes only, not lymphocytes): one crest after midnight and the other in the afternoon. These waves are independent of food intake, exercise or sleep. Swan (76) sees a similarity between his data on blood lactate in the dog and Shaw's on the leukocyte count in man.

Renbourn (81) made an extensive study of variations in a number of blood constituents, using fit army personnel as subjects. Most individuals showed regular diurnal curves for hemoglobin, hematocrit and plasma protein. The plasma protein level showed no significant changes during the usual waking hours, but after 10 P.M. dropped markedly and independently of bed rest. There were no diurnal variations in the erythrocyte sedimentation rate or in blood chloride. As some of the work was done in North India, where the diurnal air temperature range was 30 to 40° F., Renbourn points out the importance of doing the analytical work at a temperature of about 60°. Keeping a sample of blood at 104° for 4 hours produced an increase of 10 per cent in hematocrit reading and plasma protein concentration. It is possible that some reported findings of diurnal variation in blood constituents may have reflected differences in room temperature at the times the analyses were made, rather than physiological changes.

Gerritzen (82) spent 74 hours in bed, taking the same amount of water and food every hour. There was a diurnal variation in the urinary excretion of water, chloride and urea, with a peak in the early afternoon. Gerritzen concluded that not only the liver and kidney, but "all the organs of the human body function rhythmically", in accordance with a universal biological law.

Kroetz (83) measured the minute-volume of the heart in normal subjects, kept awake in bed, with little food or drink. Highest values were obtained between 6 P.M.

and midnight, lowest, at 4 to 6 A.M. Under the same conditions, the highest venous blood pressure was in the evening, lowest in the morning; vital capacity of the lungs was greatest in the afternoon and least at 2 A.M.; and the foot and lower third of leg volume rose gradually during the customary waking hours, dropping between 1 and 9 A.M. The range of diurnal fluctuations varied from 15 to 25 per cent.

Fragmentary data are available on changes in the basal metabolic rate during the morning hours. Berkson and Boothby (84) repeatedly ran two to nine tests, all before noon, on 19 adults. The composite curve shows an initial slight decline, followed by a relatively marked rise. Unfortunately, no concomitant body temperature figures were given, but it is more than probable that the temperature curve would parallel the metabolic one.

Similarly, one can trace, along with the diurnal upsurging of the body temperature, a rising cutaneous sensitivity to tetanizing faradic current, expressed as the reciprocal of the threshold stimulus, starting in the morning and running into the afternoon (85). If muscular relaxation, during the morning, leads to a drop in body temperature, there is a simultaneous decrease in cutaneous sensitivity. On the other hand, diurnal variations in acuity of olfaction have been shown by Goetzl and Stone (86) to be connected with hunger, rather than with the time of day: there is a precibal increase in sensitivity to odors.

In a breakdown of the hourly incidence of occurrence of 39,929 epileptic fits, Griffiths and Fox (87) discerned a triple wave, with the main crest at 6 to 7 A.M., and lesser ones at 11 A.M. to noon and at 11 P.M. Sleep, especially the times of its onset and termination, seems to be the most important factor in the determination of these maxima of attacks. In an analysis of the hourly distribution of 9954 cases of onset of labor by Kirchhoff (88), there was a definite maximum at 8 P.M. to midnight, with 21 per cent, and a minimum between 9 A.M. and 1 P.M., with 13 per cent. An idealized sinusoid curve of this distribution has a crest at 11 P.M. and a trough at 11 A.M. Kirchhoff quotes data from Zangemeister, which show a maximum also in the four hours preceding midnight, but a minimum in the succeeding four hours, when Kirchhoff's own data are close to the maximum (19.6 per cent). There is agreement, then, only on the hours of the greatest frequency of the onset of labor. By contrast, the incidence of actual births, according to Kirchhoff, is the same at all hours, but here, too, he quotes Lane and Jenny as finding a maximum occurrence at 2 to 5 A.M., and a minimum in the corresponding hours of the afternoon, with a difference between the two of 40 per cent (17000 vs. 12000). Petersen (10) plotted Marbe's studies on data collected at Wuerzburg, in which the greatest number of births took place at 1 to 2 A.M., but the fluctuations from hour to hour were too extreme to indicate a distinct diurnal variation.

In a repetition of Carleton's study (78), Cooper (89) determined the percentage of cells showing mitosis in 5000-cell counts in the normal skin of the prepuce, removed by circumcision, at intervals of one hour or less, throughout one 24-hour period. On the basis of skin samples from 57 newborn infants, there was a significant difference in the average number of dividing cells between the 22 samples obtained at noon to midnight (23.41 cells) and the 35 between midnight and noon (13.29 cells).

## PERIODS OF MORE THAN 24 HOURS: SEX CYCLES

There is an extensive literature, including reviews and monographs, on the primary and secondary morphological changes and physiological events that characterize the sexual cycle, and the hypophyseal-ovarian interrelationships that govern its successive phases. This review will therefore be limited to associated or concomitant behavioral and functional periodicities, as well as the timing, duration and modification of the cycle itself, which may vary from a few days to a whole year.

*Sex Cycle in Animals.* There is a marked variation in the activity of the female albino rat during the successive phases of the estrus cycle, with maximum activity at the peak of the cycle coinciding with the period of sexual receptivity (12, 39, 55, 90, 91), but Colton (39) could detect no such cyclical changes in the activity of the female wood rat. The duration of the rat's estrus cycle is usually given as 4 to 4½ days, but the height of activity tends to be superimposed upon the diurnal rhythm, according to Hemmingsen and Krarup (56), leading to a midnight maximum at 4-day intervals. These authors could shift the maximum to noon by reversing the lighting conditions and thus the diurnal cycle of activity. Browman (55) obtained similar results, but was able, in addition, to change the onset of estrus in blinded rats, and in normal rats kept in continual darkness, by varying the room temperature in a diurnal manner. The onset of estrus, under such conditions, occurred at the end of the cool 12-hour period. Alternating the temperature on the same schedule, but keeping the light on continuously, caused a prolongation of the cornified stage of the vaginal epithelium of normal rats, making the duration of the cycle five days, instead of four. Slonaker (91) noted a marked increase in activity of rats during early pregnancy (up from 40-50 per cent of the total time to 73), but a decrease in late pregnancy (down to 33 per cent).

The sow more than doubles its 24-hour activity during estrus, but is especially restless at night (47). In one case the number of steps taken by a sow in heat during the night was nearly seven times greater than usual (2243 as compared to 346).

Rogers (92) followed the variations in the differences in electrical potential between the vagina and symphysis pubis, in a number of sexually mature rats, over 2 to 5 consecutive estrus cycles. The differences were most marked during late estrus, but all cyclic variations could be abolished by ovariectomy and reinstated by injections of appropriate amounts of theelin.

Bissonette (8) reviewed the literature on sexual photoperiodicity, usually exhibited by seasonal breeders. In most cases, it is the increase in the proportion of daylight during the spring that is responsible for the development of the gonads in both males and females. In starlings it is possible to achieve complete spermatogenesis and maximum size of testes in 4 to 6 weeks, any time between November and May, by artificially lengthening the daytime hours. This also applies to ferrets, juncos (snow birds), and turkeys, but sheep go into estrus when the days get shorter, thus dropping their young in the spring. On the other hand, variations in length of days have no effect on the sex cycle of the stickleback (a fish), the 13-lined ground-squirrel, or the guinea-pig. The latter, it will be recalled, shows no diurnal variation in activity (48). The stickleback responds to changes in environmental temper-

ature, while the hedgehog is affected by temperature and length of day. Humidity, rainfall and food supply have also been found to modify the sex rhythm of certain species.

Kendeigh (93) tested Rowan's hypothesis that it is the longer wakefulness and activity, indirectly induced by longer days, rather than the light itself, that furthers the development of the gonads in birds. Increasing the daily period of activity of English sparrows, by placing them in rotating cages, without added light, had no effect on the gonads of either males or females.

A peculiar seasonal difference in the egg production of the domestic fowl was described by Winchester (94) who also collected an extensive bibliography on seasonal metabolic and endocrine rhythms. During the first productive year (in the pullet) maximum egg-laying occurs in November and December, which are precisely the months of minimum production for the mature hen. The author concludes that "egg production of pullets may be governed by endocrine factors limited to a greater degree by age-changes than by environmental conditions which appear to be limiting factors for production of 2-year old and older hens."

Bissonette (95) demonstrated that the gonad-stimulating effect of light upon the ferret is accomplished through the eyes, as two females, whose optic nerves had been cut, did not respond to increased exposure to light. Further, impulses from the eyes are transmitted to the anterior lobe of the hypophysis, as hypophysectomy likewise prevented the recurrence of estrus in this species.

Experimental induction of spawning in the brook trout, *Salvelinus fontinalis*, in August or September, instead of December, when it usually occurs, was accomplished by Hoover (96) through an increase in 'daylight' by one hour per week, for eight weeks, beginning in February, then gradually decreasing the length of 'day' to normal. Whether the preliminary lengthening of the 'day' was necessary for the premature spawning has not been determined.

*Menstrual Cycle in Women.* Among the physiological concomitants of the primary events of this cycle, body temperature changes have attracted the attention of investigators since 1870, as related in the historical review by Barton (97). In general, there is a slow decline in body temperature during the preovulatory phase, with sometimes a steeper drop just prior to ovulation. The latter, however, is followed by a sharp rise to a flat or slowly ascending plateau, and a precipitous fall one or more days before the onset of menstruation. Basal rectal temperatures were usually taken, but, like Cullis, Oppenheimer and Ross-Johnson (98), we found that the evening temperatures were as good as morning ones, and mouth temperatures could be substituted for rectal (67).

King (99) reported a general parallelism between basal body temperatures and heart rates during the menstrual cycle, but Rubenstein (100) and Altmann, Knowles and Bull (101) failed to detect any definite pulse curve. Our data on heart rates (67) offer a clue to the failure of recent investigators to detect a menstrual heart rate wave. The heart rate varies with the body temperature for a part of the menstrual cycle, and against it for another part. Immediately after ovulation, the body temperature and heart rate rise sharply, but a day or two later, the heart rate falls almost as much as it rose, whereas the body temperature continues on its upward course,

as described earlier. This produces a characteristic crossing of the two curves, indicating that ovulation is definitely over for the particular cycle. Later there is a second rise in the heart rate, with another drop, concurrent with the premenstrual fall in body temperature. There is thus a single body temperature curve, but often two distinct heart rate curves in the course of one menstrual cycle.

The basal metabolic rate is low during menstruation, but a second low occurs at the time of ovulation, according to Hitchcock and Wardwell (102). This was confirmed by Rubenstein (103) in 15 women, in which he also established a complete parallelism between variations in basal metabolism and those in basal body temperatures, at least for the preovulatory phase of the cycle. Both reach their lowest points at the time of ovulation, as determined by the vaginal smear method.

Burr and Musselman (104), and later Barton (105) and Altmann, Knowles and Bull (101), have measured the variations in the electrical potential difference between the two index fingers during the menstrual cycle. All reported a marked rise in voltage in the preovulatory phase, with a peak at the time of ovulation. Barton (105), in a study of 79 women, from all walks of life, over a total of 238 cycles, detected bioelectric peaks in every decil of the menstrual cycle, sometimes more than one peak in the course of the cycle, and cycles without discernible peaks. But the most frequent occurrence of peaks was at ovulation time. Altmann, Knowles and Bull (101) obtained a better agreement between the vaginal smear index of ovulation and the bioelectric one (85 per cent of the cases) than between the latter and the basal rectal temperature minimum (58 per cent).

Parmenter (106) could detect "no consistent unidirectional polarity of the right index finger, the potential difference of this member fluctuating both in magnitude and polarity from day to day". Snodgrass and Davis (107, 108) found the cause of these erratic results in the temperature differences of the two fingers, warmth making a finger more positive electrically, but others (101) did not obtain a good correlation between electrical potential differences and differences in temperature of the fingers.

Many studies were made of the variations in emotional states and activity during the menstrual cycle, usually by the questionnaire method. McCance, Luff and Widdowson (109) collected data on 167 women over a period of 4 to 6 months, a total of 780 complete menstrual cycles. Fatigue and depression were greatest during menstruation, while sexual feeling was most intense and incidence of elation highest, immediately following menstruation, with a secondary peak at about the time of ovulation. Altmann, Knowles and Bull (101), from data obtained on 10 women and comprising 55 menstrual cycles, observed "as the most universal and conspicuous reaction, an outburst of physical and mental activity before the onset of menstruation, coupled with high tension and irritability and preceded or accompanied by depressions. Another high in activity was discovered to dominate the ovulative phase of the cycle, but this type of activity was free from nervous tension and generally bore the character of elation."

The determination of the occurrence of ovulation has been a subject of many investigations. Its practical importance lies in the application of this knowledge, on the one hand, to increase the probability of pregnancy in cases of subnormal

fertility, and, on the other, to avoid pregnancy (birth control). Of the various methods now available, some (vaginal smear, basal metabolic rate, bioelectric potentials) require laboratory facilities, while body temperature (oral, axillary or rectal; basal, evening, or mean) can be applied by the subject herself, under home conditions, and has been found satisfactory by Davis (110) and many others. Especially when combined with heart rate data which can also be obtained by the subject without laboratory equipment, body temperature variations make it easy to pick the exact day of ovulation.

There is a great variability in the position of the ovulation day (67, 97, 101), particularly the way it usually is counted, i.e., from the beginning of the previous menstruation. If, however, one determines the interval between ovulation and the succeeding menstruation, the variability is smaller and an individual characteristic makes its appearance. Thus, our data (67) on three subjects show probable ovulations, on the average, 16.3, 13.8, and 10.8 days before the onset of menstruation, with corresponding individual ranges of 14 to 18, 11 to 16, and 8 to 13 days.

The duration of the whole cycle also varies from person to person, and in the same individual from time to time. In one study of 900 cycles (109), the length varied from 16 to 44 days, but 45 per cent of the cycles were from 26 to 28 days; in another (101), comprising 55 cycles, the range was 23 to 61 days. In a record of 110 cycles, kept by one woman for eight years, the range was only 25 to 31 days, with 50 per cent of the cycles 27 or 28 days.

Among the various exogenous influences on the time of the onset of menstruation, and thereby on the duration of the cycle, is the menstruation of another woman (relative, roommate) in the immediate proximity (67). In such situations, diverging times of menstruation are often gradually shifted until they coincide, or nearly so, with each other.

Lunar influences on the onset of menstruation have been postulated by several writers, following the lead of Arrhenius. Petersen (10) quotes statistics to the effect that menstruation frequency is accentuated at the new and full moon.

A solar effect on the menstrual cycle can be discerned in the tendency for menstruation to begin on certain dates two or more years in succession. Siblings are sometimes born on the same or close dates, several years apart. Riebold (111) introduced the notion of a 'physiological week,' as a biological unit, menstruation occurring in a multiple (3 or 4) of such weeks. The duration of the week is fixed for each person, but may vary in different individuals from 6.5 to 9.1 days, making the year equal to from 56 to 40 weeks. Menstruation follows ovulation by two physiological weeks. One can determine the length of the physiological week of a mother, by determining the differences in the birth dates of her children, irrespective of the years.

Seasonal influences on human reproduction will be discussed under annual periodicities.

#### OTHER PERIODS OF MORE THAN 24 HOURS

The duration of these periods varies from a few days to many years, and publications dealing with them will be considered under two headings: laboratory or

observational, and statistical or historical. No reflection is intended on the quality of the data gathered by the second method, nor on the validity of the conclusions drawn from them.

*Observational Data.* As already mentioned, a 6-day feeding cycle has been observed in bed-bugs (27), the diurnal light-darkness alternation being superimposed and thus somewhat modifying this rhythm. Unlike cockroaches, bed-bugs come out of their crevices at all times, but much more so at night. The explanation of the maxima of activity—before midnight for cockroaches, after midnight for bed-bugs—probably lies in the difference in their feeding cycles. Cockroaches feed on a 24 hour schedule, and hunger brings them out as soon as it gets dark. Bed-bugs are just averse to light, rather than completely inhibited by it, and their night emergence is more symmetrically distributed with respect to darkness.

Richter (112) noted a regular cyclic fluctuation in the sleep characteristics of three psychotic patients, the intervals varying from 4 to 6 days. Quoting from Kunze and Riesser, de Rudder (113) discusses the correlation between a 5- to 6-day periodicity in the phosphate content of rabbit muscle and a corresponding variation in the audibility of 10-meter waves in radio-telegraphy.

Weekly periodicities have been reported for body temperature and heart rate in man (67), a week-end low corresponding to relative inactivity, and for the blood pH (with Sunday crests) of three adult male triplets, studied for several weeks by Petersen (10). The latter went so far as to declare that "one might consider the possibility that the human group selected the 7th day of rest because of an existing underlying biochemical rhythm!"

Reimann (114) described 6 cases of periodic disease: 3 of fever recurring at intervals of 7 to 22 days, 7 days, and 14 to 28 days for 3 to 11 years, and one each of neutropenia, arthralgia and myasthenia, with periods of 20 to 22, 14, and 14 to 21 days, respectively, and durations of 2 to 3 years. In addition, he collected from the literature accounts of 12 other cases, practically all of which were characterized by a time interval of 7 days, or a multiple of 7 days, in the reappearance of the symptoms. Of the 18 patients, 15 were males, one of the latter suffering from a swelling of the breast every 28 to 56 days. Reimann's conclusion is that most of the symptoms "recur independently of any known natural rhythmic fluctuation and probably signify some obscure intrinsic cycle in both sexes."

Hersey (115, 116) kept a record of day-to-day variations in moods and emotions of male factory workers and found them to undergo a wave-like oscillation every 4 to 6 weeks. The periods were shorter for younger than for older workers. Average values in weeks for the following categories were: extroverts—5.52; introverts—4.38; intelligent—5.45; dull—4.67; aggressive—5.43; submissive—5.24; married (mean age of 32)—5.21; single (mean age of 26)—4.52. Older, extrovert, intelligent, aggressive and married workers thus showed longer mood cycles than their opposites. Women, according to Hersey's observations, may be affected by both the menstrual cycle and the sort of cycle seen in the male, the two at times criss-crossing.

A spontaneous activity cycle of from two to six months was observed in albino rats by Slonaker (117). It is common to both sexes and extends into old age. Lack of synchronization among several animals shows that it is not a periodicity associated



with changes in temperature, pressure or humidity of the air. During its life-time, the albino rat has at least three waves of activity of 200 or more days, and on these are superimposed shorter fluctuations which vary in number and duration in different animals.

Seasonal or animal periodicities may be related to variations in light, temperature and humidity. A particular set of meteorological changes will have different effects, however, even on closely related forms. Beginning in October, hibernating frogs (*Rana pipiens* and *Rana catesbeiana*), according to Holzapfel (118), show a gain in weight, a darker color of skin, an increase in muscle tonus, but a drop in blood volume, erythrocyte count and hemoglobin. From March on, these changes are reversed. True dormancy can be obtained in winter only. Non-hibernating frogs (*Acris*) do not respond to seasonal environmental changes. Seasonal variations in hibernating frogs also occur when temperature, humidity and food supply remain unchanged, which leads the author to conclude that "hibernation appears to be one phase of the seasonal cycle which is an intrinsic function of the animal itself." Among mammals, the hedgehog exhibits a seasonal variation in the ease with which a fall in air temperature will induce it to hibernate.

In man, an annual periodicity in heart rate, as determined nightly on himself for five years, was reported by Coste (119), with a low early in the summer and a high in the first months of the winter. Grollman (120), on the other hand, could detect no seasonal differences in either heart rate or output, under basal conditions. Our data (67) reveal a distinct annual periodicity in the mean daily heart rate, the maximum occurring in the summer. Basal values, however, do not vary seasonally, which is in agreement with Grollman's figures. Coste's results can easily be explained by his arbitrary rejection of all pulse counts of over 79 per minute. In the summer such counts are more frequent than in the winter, and leaving them out resulted in an inversion of the annual heart rate curve.

Pulse variations represent a coupled periodicity, as they depend directly on body temperature, which shows the same kind of seasonal fluctuations (67). During the heating season, body temperature varies independently from external air temperature, as one lives in an 'artificial' climate, in which the day-time air temperature is always about 70° F. In the summer, on the contrary, the inside and outside air temperatures are nearly the same, and then it is easy to demonstrate a direct relationship between the mean daily figures for the air temperature, on the one hand, and body temperature, as well as heart rate, on the other. In one subject, for example, when the daily mean air temperature fluctuated between 60 and 64° F., the mean daily body temperatures averaged 97.98° F. and the pulse, 79.4 beats per minute (67). When the air temperatures were between 85 and 89°, the corresponding body temperatures rose to an average of 98.32° and the heart rates, to 86.3. Thus, an increase of about 25° in external temperature occasioned, in this subject, a rise in body temperature of 0.34°, and in heart rate, of 6.9 beats per minute, or about 20 beats variation per degree F. increase in body temperature.

Basal metabolic rate, like body temperature and pulse, shows no marked seasonal variation, just because it is basal. However, Gustafson and Benedict (121) reported a tendency to a low level in the winter and a higher one during spring and summer in

20 young women, tested from October to June. Hitchcock and Wardwell (102), also in 20 women, found low basal metabolic rates in the winter and spring and high ones in summer and autumn.

A seasonal variation (September to March) in blood lactate in the dog was noted by Swan (76). There was no direct relation with the mean temperature of the month, but rather with the temperature mean deviation, i.e., the dispersion of the daily temperatures from the monthly mean temperature. During November this dispersion was greatest, and the blood lactate figures, highest. Petersen (10) found the  $pH$  levels of venous blood of man to be lowest (7.37) in the middle of April, highest (7.46–7.47) in summer and autumn. Renbourn's data on blood hemoglobin, hematocrit, plasma protein, erythrocyte sedimentation rate, and blood chloride (81) showed a significant seasonal variation in most individuals, higher values prevailing during the hot season. Certain sleep characteristics manifest a definite seasonal periodicity (7). Both body temperature and motility are higher in the summer and autumn than in the winter and spring. That environmental conditions are largely responsible was shown by recording for three years the night-to-night motility of two sisters, six and four years old respectively, at the beginning of the period of observation. Although one of the girls was consistently more quiet than the other, there was a parallelism between their annual motility curves not only from season to season, but from year to year (the second year values lower, and the third year higher, than the first).

Recollection of having dreamed during the night also changes with the season of the year, being highest in the spring and lowest in the autumn, or varying in the opposite sense from motility. It is easier to fall asleep during one season than another, but in this case the annual curve is bimodal, with spring and autumn more favorable than summer and winter. The number of hours slept per night and the feeling of being well rested on getting up in the morning do not fluctuate significantly during the year.

There is a seasonal variation in the rate of growth, as determined by repeated measurements on the same individuals. Hitchings and Fitz (122) weighed 20 boys once a week and measured their height every three months. Over 90 per cent of the total increase in weight for the year occurred from June to December. Weight fluctuations were greatest during the period of minimum growth. In 17 out of the 20 boys the increase in height was either uniform throughout the year, or varied with the increase in weight.

*Data from Vital Statistics and Meteorological and Astronomical Tables.* The detection of tidal and diurnal periodicities by examination of statistical data has already been mentioned (10, 19, 88). Weekly, lunar, annual, and sun-spot variations have also been detected by this method.

Studying the weather tables for Chicago, between June 19 and July 31, 1940, Petersen (10) found a precipitation crest on Fridays, highest air temperatures on Sundays, and lowest on Thursdays. The weekly  $pH$  crests in the triplets under his observation during that period, it will be recalled, occurred on Sundays. Further correspondence between a weekly weather periodicity and biological phenomena was obtained by Petersen in a 'striking 7-day rhythm' of the vital index, or the ratio of conceptions to deaths. In scarlet fever statistics for 1934–35 he discerned a weekly

periodicity in the appearance of the rash, with peaks on Fridays. He sums up his findings thus: "The Vital Index is related to weather. The onset of the scarlet rash is related to weather. The Vital Index reveals a weekly rhythm; the scarlet rash reveals a weekly rhythm; the weather reveals a weekly rhythm."

Many weekly periodicities, according to Petersen, are really reflections of lunar variations. When he arranged the statistical data on scarlet fever on a lunar axis, or as 14 days before and 14 days after the full moon, he found that scarlet fever trends were significantly related to blood pH, the coefficients of correlation between the two being  $-.510$  for 1934, and  $-.477$  for 1935. Also, on the basis of the lunar axis, the correlation coefficient between blood pH and vital index was  $-.891$ . Epileptic attacks likewise show a lunar periodicity. With respect to deaths there is a selective sex difference, male deaths being numerous, and female deaths few, during full moon. Deaths from cardio-vascular diseases and from tuberculosis occur in greater numbers after full moon than before. Deaths by suicide (Chicago statistics for 1930, 1936, and 1937) show a minimum at full moon, but "more slender types apparently commit suicide at the period of the new and the full moon."

Fanciful notions of near-lunar biological rhythms have been advanced by Fliess (123), Tuercke (124) and others, and are mentioned here to warn the reader against wasting his time on their books. Fliess maintained that men have a 23-day rhythm of body functions, and women a 28-day one. Tuercke, while accepting Fliess's classification of rhythms for bodily functions, postulated the existence of a 33-day rhythm for intellectual activities. The publishing house of *Bioritmo* in Zuerich has put out a list of publications by Fliess, Tuercke and several other writers, all of them dealing with the mystery and significance of rhythms in human life. Sarkissiantz (125) and Aebly (126) demolished these claims by showing up the flimsy, unscientific grounds on which they were based. Even Riebold (111), whose own 'physiological week' thesis is a statistical tour de force, rejects Fliess's 23- and 28-day rhythms.

Annual or seasonal periodicities, with highs in late winter and spring, for puerperal sepsis and scarlet fever have been gleaned from statistical data by Petersen (10). He relates the maximal incidence of scarlet fever to a seasonal decline in the pH of the blood. Likewise, the birth figures for Chicago and New York lead Petersen to conclude that more males are conceived in the early part of the year, more females, in "the relatively stable alkalotic summer and autumn." Conceptions and deaths of geniuses have their maxima in April and in April-May, respectively.

Huntington has published several books on the influence of climatic changes on man. In "Season of Birth" (127) he discussed the seasonal periodicity of reproduction in man, "an inheritance from a very ancient time, when relatively few children survived unless they were born at the best season." In the determination of the best season, temperature is the dominant factor, although other climatic conditions may play a subsidiary part. According to Huntington, there are two climatic optima: for physical activity, an average daily temperature of 60 to 65° F., and for mental vigor, 39 to 54° F. His main conclusions are:

That the conditions governing season of birth among our primitive ancestry apparently explain the mental as well as the physical optimum of temperature. Our bodies apparently function best at the temperature which prevailed in primitive times at the mating season, for children conceived at that season were best able to survive. This gives us a physical optimum. Our minds function best

at the temperature which prevailed in primitive times at the normal season of birth. At that time, more than any other, the survival of the new generation depended upon the alertness of the parents. Thus a mental optimum became established at a temperature lower than the physical optimum.

On the statistical side, Huntington quoted data to prove that eminent people tend to be born in great numbers in January, February, and March, but are "conceived in maximum numbers at a temperature some 6 to 8° F. lower than that at which the maximum number of conceptions takes place among people in general." Why that is so Huntington did not know.

Fitt (128) reviewed data from 38 studies on 8 to 15,000 children in 12 countries, on the seasonal variations in weight gain. Most of them show autumn maxima. Fitt's own findings on 355 boys and girls, 7 to 15 years of age, gave an autumn-winter maximum, though not as preponderant as that of Hitchings and Fitz (122). An analysis of data for height increases, mostly from the same studies, revealed a maximum in spring and summer, or the opposite type of seasonal curve from that for increases in weight.

Fitt plotted figures for seasonal fluctuations in muscular capacity, mental ability (memory, cancellation, scholastic achievement), children's illness, juvenile delinquency, mortality, suicide and insanity rates, physical and mental characteristics, as related to month of conception, and concluded that the year might be divided into two parts. Autumn and winter are a period of minor stress, of relative ease, when the organism holds its own, and work is not hard, while spring and summer comprise a period of major stress, when the quality of performance suffers and there is a greater tendency to breakdown.

The sun-spot activity, which has a period of  $11\frac{1}{2}$  years, has been linked by Petersen (10), either directly or through its effects on temperature and weather conditions, with a variety of biological fluctuations. Among these are the sex ratio of the newborn in Sweden, the tendency to slenderness among the newborn, the birth rate of twins and geniuses, and the incidence of several infectious diseases (cholera, puerperal sepsis, erysipelas, meningitis, small-pox, measles, lobar pneumonia). The fluctuations in resistance to some infections may be related to the pH of the blood, which, according to Petersen, shows not only a weekly, lunar, and annual, but also a sun-spot periodicity. This first demonstration of the existence of a long-range biochemical tide "brings us closer to the problem of the why and how of changing human (and general biologic) reactions, that are in some fashion related to the sun-spot cycle—changing physical and mental resistance—changing trends in evolutionary and revolutionary forces." Petersen goes as far back as Egyptian chronology to bring out the occurrence of significant social events in tune with solar timing. Among recent events, he points to the Russian revolutions of 1905 and 1917 as coinciding with crests of sun-spot activity. The Fascist and Nazi revolutions of 1922 and 1933, respectively, took place in periods of minimum sun-spot activity. Petersen does not mention them, but one should surmise that sun-spots affect the Latin and Teutonic temperaments in the opposite sense than they do the Slavic.

#### PHYSIOLOGICAL MECHANISMS

The difficulty in correlating the various views and opinions on how periodic biological phenomena are established and maintained lies in the confusion of terms

used. It is hoped that the classification proposed in the beginning of this review will clarify, rather than muddle, the situation. If adopted only for the purposes of discussion, it will be seen that *periodicities*, as time-associated physiological changes, need no particular mechanism, since they are directly related to changes in the external environment or, when coupled with another physiological variable, in the internal environment. Thus, the heart rate of the frog may show a diurnal periodicity with the variations in the external temperature, and the heart rate of man will fluctuate diurnally because of variations in body temperature. *Cycles* have different mechanisms, from the pacemaker of the heart discharging intermittently through the operation of a refractory period, to the chain of events in the female sex cycle, brought about by the interaction of endocrine systems. Cycles, while essentially inherent, can be modified by internal (nervous, endocrine) and external influences, in the latter case, to use Piéron's terminology (129) becoming synchronized with an external periodicity. Sleep and wakefulness are alternating phases of an innate rest-activity cycle, which by experience or training becomes synchronized with the astronomical periodicity of night and day. All *rhythms* are therefore synchronized cycles, but the reverse is not true. A synchronized cycle is not a rhythm, unless it persists under artificially maintained uniform environmental conditions. For instance, the adaptation of the sex cycle to the annual periodicity of the sun represents a synchronized cycle, but, as shown by Bissonnette (95) and others, it does not persist when the natural external periodicity of lengthening days is modified in the laboratory. The synchronization of a cycle may result from either individual or racial experience. Park (9) holds "that biological rhythms have been selected by the rhythmic [in our terminology 'periodic'] environment, operating upon mutations which have a positive adjustment value. Protoplasmic inheritance is not necessarily limited to morphological features." A similar stand is taken by Woodbury (130) who developed the 'periodic-response' theory of the origin of animal migrations. According to this theory migration "has arisen in many animal groups and at various points in time as a response to environmental periodicities which at first were merely conditioned behavior patterns but which, through mutation and natural selection, have become so fixed in heredity of some animals, that they recur independently of the environmental stimuli because of hereditary mechanisms." As regards the mechanisms of the animal synchronization of the sex cycle, we have evidence (95) of the involvement of both the nervous system (through the eyes) and the endocrine system (through the hypophysis). Woodbury (130) sums it up by saying that "a combination of stable nervous system modified by fluctuating glandular secretions probably provides the metabolic mechanism."

It is hard to carry out experimental investigations with the year as a unit of time, and the question of the existence of annual rhythms, as distinguished by demonstrated coupled periodicities and synchronized cycles, will have to remain unanswered for the present. Using persistence as a criterion, we are therefore left with a few examples of tidal (both 6-hour and 14-day) rhythms, and a great number of observations on, and attempted modifications of, diurnal rhythms. In discussing the mechanisms involved, a distinction must be made between establishment and main-

tenance under natural conditions and modification and persistence under artificial conditions.

*Establishment and Maintenance of Diurnal Rhythms.* The synchronization of a natural cycle with the diurnal periodicity of night and day may be a time-conditioned reflex, as suggested by Piéron (129), but this explanation cannot apply to the diurnal color rhythm of the lizard, which is not maintained after hypophysectomy (36). A metabolic cycle, however, could be synchronized by either nervous or endocrine influences, or by both in unequal proportions. Of the variations in illumination or in environmental temperature, naturally occurring on a 24-hour schedule, darkness-light is more important than cold-warmth, as shown by Kalmus (29) on *Drosophila* flies, and by Browman (50, 54, 55) on rats. Piéron (129), however, in studying the behavior of some beach flies, that bury themselves in the sand by day and emerge at night, proved that these animals had a diurnal rhythm of activity which persisted when they were left in complete darkness for 5 days. Keeping the flies in the laboratory, under natural alternation of night and day, led to a gradual deterioration of the diurnal rhythm, with complete disappearance in 6 to 7 weeks. Evidently, in this case, it was not the darkness-light alternation, but the diurnal changes in temperature (perhaps the desiccating action of the sun's heat) that was the primary cause of the synchronization. In any case, the existence of a 'metabolic clock' is shown by the effects of varying the temperature of the environment, or other means of changing the metabolic rate, on the diurnal rhythm. Wahl (131) in bees, and Grabensberger (132-134) in ants and wasps, trained to feed on a 24-hour schedule, could make the animals come out at intervals longer or shorter than 24 hours, by cooling or warming their nests, as well as by lowering or raising their metabolic rate by pharmacological means.

To take the human sleep-wakefulness cycle as an example of a fundamental metabolic variation of rest and activity, it is easy to follow the synchronization of this cycle with the 24-hour alternation of night and day in the life of a single individual. Practically from the day of birth (16,58), the baby is subject to 'acculturation' with the social environment, light, warmth, noise etc., impinging on it during the day. All of these stimulate activity, and thus metabolism, during a fixed fraction of the 24-hour period. Muscular activity raises body temperature, which increases the activity of the nervous system, the latter in turn augmenting muscular activity. In the older child and the adult there are established corticomuscular circuits, or 'feed-back' mechanisms, to use a popular term, which operate like a vicious circle. Both muscular activity and nervous activity (metabolism, body temperature, efficiency of performance, etc.), keep on rising. As the muscles get tired, not only from overt work, but from postural or tonic activity inherent in being awake, the trend is reversed. This reversal is aided by the onset of darkness, the lowering of the air temperature, and the abatement of noises, characteristic of evening in the social routine of living. When the reversed trend of activity and metabolism has reached a certain low ('drowsiness level'), it becomes harder to remain awake. Sleep occasions a further drop in all the variables, until the diurnal minimum is reached, but proprioceptive impulses from the muscles, cramped by

prolonged maintenance of certain positions, increase from more frequently 'turning over' as the night goes on. When aided by visceral impulses (from stomach, urinary bladder, etc.), and environmental influences incident to the advent of morning, it becomes more and more difficult to remain asleep. Fundamentally, there is nothing about the sleep-wakefulness cycle that inherently demands a 24-hour spacing. Its synchronization with night and day is the easiest temporal adaptation for the organism to make, once the cortico-muscular circuits have been put into operation physiologically. How much of an endocrine element there is in this synchronization is not known, but the metabolic (also temperature, circulation) rhythm can, and probably does, affect the secretory activity of such glands as the hypophysis, which may act in an auxiliary capacity in both the establishment and maintenance of the diurnal sleep-wakefulness rhythm.

*Reversal and modification* of diurnal rhythms are brought about by artificially changing the astronomical or social schedule. In man the latter is more important, and it has been possible to establish synchronizations of sleep and wakefulness with 21- and 28-hour schedules of living (7), but not 12- and 48-hour synchronizations (7, 68) as the influence of the 24-hour periodicity of night and day is too hard to overcome. In ants, Grabensberger (132) did succeed in developing time feeding habits in multiples of 24 hours, up to 5 days, as well as odd rhythms of 3, 5, 21, 22, 26, and 27 hours. Two feedings at chosen intervals were often sufficient to establish non-diurnal feeding rhythms, whereas in ants usually frequenting flowers one feeding brought on a 24-hour rhythm that persisted for 9 days. It will be recalled, however, that Johnson (38) did not succeed in establishing an artificial 16-hour activity rhythm in mice, nor Hemmingsen and Krarup (56) in rats.

*Persistence* of a rhythm must remain unexplained for the present. Gerritzen's statement (82) that it is "in accordance with a universal biological law" does not touch on the mechanism. The "relaxation oscillations" of van der Pol (135) may fit the pace-making properties of the refractory period, but not the much slower diurnal rhythmicity. We have, however, inklings that the hypophysis is involved, either primarily, as in the axolotl (29) and lizard (36), or secondarily, as in the rat (57, 75). The hypophysis can theoretically be set into rhythmic activity by diurnally interrupted darkness or by interrupted light. Aside from Higginbotham's experiments on toads (136), continuous light was found by all investigators to cause a weakening or complete abolition of the diurnal rhythmicity. Under constant darkness, on the contrary, rhythmical activity continued unchanged, in the case of Johnson's mice (137) for a record time of 18 months. As judged by the permanent night-type coloration of hypophysectomized lizards (36), light is an excitatory stimulus, but it is also an inhibitory one to the rhythm itself. Light is needed to establish rhythmicity (29), but darkness is required for its maintenance and persistence. The inhibitory effect of continuous light was studied quantitatively by Hemmingsen and Krarup (56) and by Johnson (137). The normal activity period of their rats and mice respectively, commenced later each day, rats by 2 hours, mice by 40 minutes, so that, after a number of days, the summated shift amounted to 24 hours. Johnson further demonstrated that mice, changed from a

regime of continuous light, with its daily delays, to one of continuous darkness, re-verted to the normal 24-hour rhythm of activity. As Johnson puts it, "this animal has a substantial and durable self-winding and self-regulating physiological clock, the mechanism of which remains to be worked out. In continuous light this clock continues to run, but at a slower rate. No procedure has yet been found which will consistently or dependably move the regulator to the 'fast' side." As stated earlier, Kalmus (29) and Grabensberger (132-134) did succeed in moving the regulator to the fast side in insects, but the mechanism still "remains to be worked out."

To summarize, a physiological rhythm, specifically the diurnal rhythm, is essentially a metabolic cycle synchronized with the external periodicity of day and night through the influence of variations in illumination, temperature and other environmental factors, on the nervous and endocrine systems.

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# INITIATION AND EARLY CHANGES IN THE CHARACTER OF THE HEART BEAT IN VERTEBRATE EMBRYOS

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IN DEALING WITH ADVANCES in science there is a natural tendency to emphasize the newer findings appearing in the current literature, and pass too lightly over the long series of foundational steps that preceded them and made them possible. In assessing our present knowledge as to when and how the embryonic vertebrate heart begins to beat, we should, therefore, not fail to cast at least a brief glance backward.

The unaided eyes of early observers could make of the beating heart of a transparent living embryo little more than a pulsating red fleck. With the discovery of the circulation in the adult and the development of the microscope, the embryonic heart began to be studied with new understanding. Haller (33) saw the beating heart and the moving blood in chicks as young as 45 hours of incubation, and was impressed by the inherent rhythmicity of the pulsations of the embryonic heart. Schenk (67) excised the heart of a 3-day chick embryo, kept it beating outside the body and noted its increased rate when he raised the temperature of the warm stage. Moreover, he cut the heart into fragments and noted that after a quiescent period following the operation these resumed rhythmic contractions. One of Schenk's statements (p. 111) should be quoted verbatim: "Die Bewegungen des embryonalen Herzens in einer bestimmten Periode der Entwicklung, ist eine jener Bewegungserscheinungen die vom Einflusse des Centralnervensystems unabhängig ist."

Wernicke (1876) observed that the heart rate increased during the early stages of development, and began to try out the effects of different chemical solutions on the rate of its pulsation. Fano and Badano (23) in an exceptionally penetrating study of the embryonic chick heart crushed it at the atrio-ventricular sulcus and noticed that the atrium continued to beat without interruption. The ventricle stopped altogether for a time and when it did resume beating its rate was slower than the rate of the atrium. They also cut the heart in a zig-zag ribbon and noted that the beat was transmitted along this ribbon from the atrium toward the ventricle. They commented on the peristaltoid character of the contraction of the young tubular heart and estimated the spread of transmission of the wave of contraction at 6 to 8 cm. per second. In their studies, heart movements were recorded photographically by throwing the shadow of the heart against a narrow aperture in a camera containing moving film. They also tried the effect of various gases on the rate of cardiac pulsation noting particularly the striking effects of oxygen and carbon dioxide.

Another unusually interesting paper of about the same period is that of Pickering (62). He built a special water-jacketed chamber for studying the heart action of chicks under better controlled temperature conditions than previous workers had employed. In addition to studying the effects of temperature changes he investigated the effects of drugs such as caffeine, xanthine, theobromine, digitalis, nicotine, and hydrocyanic acid. Of special interest is his report of the reversing of the direction of the heart beat by treatment with dilute hydrocyanic acid and, sometimes, under the influence of amyl nitrite or morphiae. These experiments I have tried to repeat without success, but incidental to other studies, I did see one case of spontaneous reversal of the direction of beat in a chick embryo of about 55 hours. Unfortunately, this embryo reverted to normal beat propagation before a microcinematographic record could be made. Bremer (8) records having seen two cases of similar spon-

taneous reversal of the heart beat.<sup>1</sup> These observations lend support to the reversals reported by Pickering, despite the lack of success up to the present in inducing such reversals under controlled conditions. Perhaps the most striking of Pickering's experiments, and one which has since been repeatedly confirmed, was his production of a ventricular block by exerting pressure from a small piece of silk fiber across the atrio-ventricular junction.

Thus, before the end of the nineteenth century, we find well established some of the most significant facts as to the general character of the embryonic heart beat. Its inherent rhythmicity had been remarked upon by Haller (33). The definitely myogenic character of the early embryonic heart beat had been clearly enunciated by Schenk (67). Wernicke (76) had noted the change in heart rate due to temperature changes; Fano and Badano (23) had established the fact that there was a difference in the inherent rate of contraction of different parts of the young tubular heart and that its beat was peristaltoid in character. Pickering (62) had attempted the first comprehensive study of the effect of drugs on the embryonic heart, and had made the significant observation that heart block could be caused by pressure applied at the region of narrowing between atrium and ventricle.

#### SEQUENTIAL FORMATION OF THE PRIMARY REGIONS OF THE HEART

Almost concurrently with the studies of Fano and Badano and of Pickering dealing with cardiac activity, Born (6, 7) was developing the wax-plate method of reconstruction which was destined to add many new pieces to the growing mosaic of our knowledge of the embryonic heart. Earlier workers in their observations of heart action had taken too little account of the radical changes that were occurring in the structure of the heart itself as its development progressed. The plate-reconstruction method brought an infinitely greater accuracy to our analysis of the morphogenesis of the heart. Although this review is concerned primarily with the initiation of cardiac pulsation and the early changes in the character of the heart beat, the fact that the embryonic heart does not begin at all as a miniature of the adult organ makes it imperative that we should review its early structural changes before attempting to interpret its activities. For the plan of the embryonic heart, even the chambers present, changes with age in a manner which makes physiological observations meaningless unless they are properly correlated with the changing structure of the heart itself.

Utilizing the Born wax-plate method, the structure of the embryonic mammalian heart has been carefully studied in a large number of forms. To mention but a few of the more detailed papers that deal with the critical early stages in the fusion of the paired cardiac primordia there is the beautiful work of Davis (18) on the human heart; that of Schulte (68) on the cat; Wang (75) on the ferret; Yoshinaga (79) on the guinea pig; Patten (57) on the pig; Girgis (24, 25) on the rabbit; Goss (27) and Burlingame and Long (9) on the rat. In forms below the mammalian level the fusion of the

<sup>1</sup> Gowanloch ('22) reported having induced reversals in the heart of teleosts "by the action of various chemical and physical agents during early development." Unfortunately, these observations seem to have been presented only in the form of an abstract without any details as to the age of the material or the kinds of "agents" employed.

cardiac primordia and the establishment of the early regional divisions of the heart have been studied in the chick by Patten (56), in the duck by Yoshida (78), in *Amblystoma* by Copenhaver (14) and in fishes by Senior (69) and Armstrong (2).

There are, naturally, detailed differences in the development of even closely related forms, but they do not particularly concern us in the present connection. One developmental trend that is of basic physiological importance is shared by the above group as a whole. Because this particular phase in the morphogenesis of the heart has been too often slighted in studies dealing with cardiac activity it should be reemphasized here. It concerns the sequential formation of the primary regional divisions of the embryonic heart. The embryo of primitive vertebrates is endowed with a considerable amount of stored food material and the body must develop prone on this inert mass, as if it had been split open midventrally and closely applied to the relatively enormous spheroid constituted by the food material stored as yolk. This means that certain structures such as the heart which are destined to be midventral in location are formed from paired primordia which arise on either side of the mid-line while the embryonic body still lies spread out, open ventrally, on the surface of the yolk sphere. These paired cardiac primordia can not meet each other in the mid-ventral line until the embryonic body has grown sufficiently to pull away from the yolk mass, establish a floor under the foregut, and complete the ventral body-wall. This process proceeds cephalo-caudally toward the mid-body region. This manner of completion of the ventral part of the body in the thoracic region means that the paired cardiac primordia must of necessity fuse in the same cephalo-caudal sequence. The tubular embryonic heart, therefore, has its cono-ventricular region established first, its atrial region at a somewhat later time, and its sinus venosus last of all. This sequential formation of the heart is summarized for the chick in figure 1. The formation of the heart in fishes and in amphibia is similar in its sequential character. Even in the higher mammals, which have ceased to store yolk in any quantity, the yolk-sac remains as a phylogenetic imprint on their ontogeny, so their embryos develop spread out on an empty yolk-sac, and their hearts exhibit the same paired primordia and the same cephalo-caudal sequence in their formation. This origin of the heart from paired primordia may be dramatically emphasized by procedures interfering with mid-line fusion. Cuts between the as yet unfused primordia, or extirpation of a wedge of mid-line tissue, or even pressure exerted between the growing primordia, cause the development of double hearts. Such experiments have been carried out on chicks (32), on amphibian embryos (20, 13, 22) and on rat embryos (26).

If one realizes that cardiac pulsations begin in an embryo before all the parts of the heart have been formed, the importance of the sequential nature of the fusion of the cardiac primordia, and of the resultant sequential formation of the several regions of the heart as a basis for any studies of early embryonic heart action is at once apparent. In the earliest stages of its activity the heart is essentially nothing but a primitive ventricle, with a conical discharging end. Later the atrial region is added caudal to the ventricle, and last of all the sinus venosus is formed caudal to the atrium. As each new region is added it brings tissue of different physiological properties into the complex, and the character of the heart beat shows correlated changes.

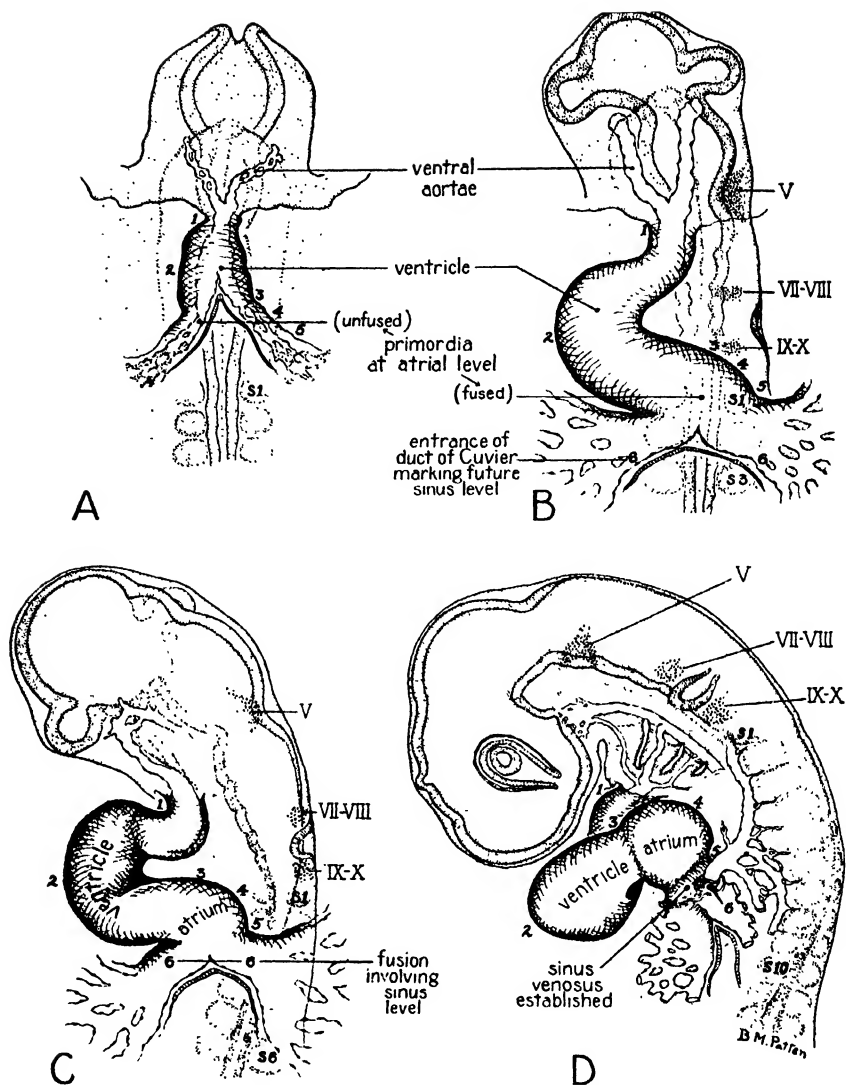


Fig. 1. FORMATION OF THE FUNDAMENTAL REGIONS of the chick heart by progressive fusion of its paired primordia. *A*, at the 9-somite stage, when the first contractions appear. The cono-ventricular part of the heart is the only region where the fusion of the paired primordia has occurred and their myocardial investment has been formed. *B*, at the 16-somite stage, when the blood first begins to circulate. The atrium and ventricle have been established, but the sinus venosus exists only as undifferentiated primordial channels, still paired and still lacking myocardial investment. *C*, at the 19-somite stage. Fusion of the paired primordia is just beginning to involve the sinus region. *D*, at the 26-somite stage. The sinus venosus is definitely established and its investment with myocardium well advanced. While schematic as to manner of drawing, these figures are based on projection outlines of actual preparations, and such structures as the somites and the cranial ganglia V, VII-VIII, and IX-X are shown in their exact relationships to serve as landmarks in following the progress of fusion of the cardiac primordia. As an additional aid in following this fusion, arabic numerals have been placed against approximately corresponding locations. The 6 is located at the point of entrance of the duct of Cuvier as determined from injected specimens, and serves as a precise indication of the level of future sinus territory. The heavy black outlines and the crosshatched contours indicate the extent to which the heart is invested by differentiated myocardium. Note especially the absence of anything like an effective myocardial layer encasing the sinus region until a considerable time after the heart has begun to beat and the blood has been set in motion.

## FIRST CONTRACTIONS IN THE MYOCARDIUM

The recent advances in our knowledge of heart action in young embryos have stemmed largely from new techniques which have made observation possible for longer periods under better controlled conditions, and from the development of recording methods of greater accuracy. Chief among these are the applications of tissue-culture methods (10, 48) to the handling of Sauropsidan embryos. Such techniques have made it possible to keep the same embryo alive under reasonably good physiological conditions for periods sufficiently long to permit observation of the changes in activity which occur as the primitive tubular heart progresses toward its adult condition. One of the most valuable of the new recording techniques has developed from the utilization of micromoving pictures of cardiac activity. Such records offer unequalled means of direct comparison of data obtained from experiments carried out on different occasions. Moreover, the possibility of controlling the time factor by speeding up the rate at which growth processes appear on the screen, and conversely, the use of the 'slow-motion' technique for the analysis of processes too rapid to follow with the unaided eye, make micromoving pictures peculiarly valuable in the study of the embryonic heart.

Because of the widespread use of chick embryos as a basis for both morphological and experimental studies it is not surprising that they were the subjects of the first detailed observations on the beginning of cardiac activity. Even in this much-studied form it was not until work with tissue-culture methods had paved the way that continuous observation of the same embryo for considerable periods of time made it possible to see the very first signs of myocardial activity. The first record of the actual beginning of cardiac contraction as it can be seen in living embryos kept under continuous observation *in vitro* appears to have been made by Sabin (66). Incidentally to her beautifully detailed observations on the origin of the blood vessels of the chick, she noted the time at which the first beating of the heart commenced and that this first pulsation was not in the venous part of the heart, but in the ventricle. She says (p. 255):

"The very first beats of the heart can be made out in these hanging drop specimens. They occur at the stage of 10 somites and always in the same position. The first twitching is along the right margin. . . . It is interesting to note that there is no movement whatever in the vein, the entire twitching being confined to the ventricle proper. . . . The beat is at first slow but rhythmical, and gradually involves the entire wall of the ventricle, spreading from the posterior to the anterior end."

These observations Sabin herself did not pursue beyond putting them thus on record. They were, however, repeated and confirmed by Johnstone (40, 41) in two striking papers which we shall have occasion to consider further in connection with the early changes in the location of the pace-making center.

Olivo (52, 53) studied the chick heart intensively at the time it first exhibited contractile activity. Like Sabin, he reported the first contractions as involving only very small areas of the myocardium. In agreement with Sabin, also, he regarded the beating as rhythmic from the outset although its rate was at first no more than 8 per minute. Olivo reported the first contractions as occurring in 9-somite embryos which agrees, well within the range of individual variability, with Sabin's findings.



More difficult to reconcile is Olivo's statement that the first contractions appeared on the left more frequently than on the right.

Patten and Kramer (59, 60), having designed and constructed an apparatus especially adapted for work with living embryos, made microcinematographic records of the first heart beats as seen in chick embryos cultured *in vitro*. By making preparations of specimens somewhat younger than the 9 to 10-somite stage at which Sabin and Olivo had noted the first pulsation, it was possible to keep embryos under continuous observation during the period when the beating of the heart was due to commence. In such preparations the first contractions of the myocardium were seen to occur in the tubular heart when it had reached the stage of development (9 to 10 somites) indicated in figure 1 A. The only region of the heart which has at this time been established by fusion of its paired endocardial primordia and their complete investment by epimyocardium is the cono-ventricular portion. The atrium is represented only by paired endothelial tubes lying either side of the anterior intestinal portal and these tubes have as yet acquired little more than a partial covering of the splanchnic mesoderm which will later become differentiated into their epimyocardial mantle. The sinus venosus at this stage, is represented only by the paired endothelial tubes, diverging toward the yolk-sac, which show neither a recognizable local specialization setting them apart from the omphalomesenteric veins with which they are continuous, nor any differentiated myocardial investment.

In agreement with Sabin (66) and Johnstone (40, 41) the moving picture records of Patten and Kramer show the first contractions occurring along the right margin of the ventricle. The contractions were exceedingly small in amplitude, the earliest of them appeared to involve only a few cells at a time, and the impulse did not seem to spread by conduction. Superimposed tracings made by pulling the film, a frame at a time, through an enlarging apparatus, showed that these first flickers of contraction were not limited to any single sharply localized area. They occurred, rather, in unpredictable sequence in slightly different places along the right side of the ventricular myocardium. As the preparations were watched there appeared to be a gradual spreading of the areas involved in the contractions, until all of the right side of the ventricle began to contract. Soon thereafter the entire ventricular myocardium began to contract synchronously.

Sabin, Johnstone, and Olivo all described the first beats as being rhythmic. Patten and Kramer found the earliest contractions appeared intermittently. If observed during a period of activity the local fibrillar contractions could be seen to occur in rhythmic series as described by Sabin, Johnstone, and Olivo, but in observations extending over long continuous periods there were intervals of quiescence followed again by another series of pulsations. This sort of intermittent rhythmicity is sometimes spoken of by physiologists as 'Lucciani grouping.' Nordmann and R  ther (51) regarded pauses in the beating of their cultures of embryonic heart muscle as signifying exhaustion, and it is possible that the pauses in rhythm reported by Patten and Kramer were indicative of unfavorable culture conditions. Certainly pauses in rhythm, once cardiac pulsations are well established, should be so interpreted. These very young embryos appeared, however, to go on to regular rhythmic beating suggesting that the culture conditions were at least reasonably favorable. Whatever

interpretation future work may place on it, the phase of intermittent activity lasts but a brief time during which the periods of beating become progressively longer and the intervals of quiescence shorter, so there is rapid progress toward the establishing of sustained regular rhythmic pulsation.

Armstrong (2) has carried out exceedingly interesting studies on the embryonic heart of the small marine teleost, *Fundulus*. His work shows that some of the developmental processes in this form differ in their timing when compared with corresponding processes in the embryos of higher vertebrates. Most striking is the fact that in *Fundulus* the myocardial primordia fuse in the mid-line before endocardial tubes have been formed. Thus the primordial heart is at first a solid cone of myocardium without any endothelially lined lumen. When the first cardiac pulsations occur at the 13-somite stage (personal communication) the heart still lacks any lumen. By the 16-somite stage endothelial cells have been differentiated within the myocardium and with their belated appearance the embryonic heart of *Fundulus* is essentially similar to the tubular heart of other young vertebrate embryos.

It seems probable that the solid myocardial cone where pulsation first appears in *Fundulus* is homologous with the conoventricular portion of the chick heart, although Dr. Armstrong tells me that the sequential formation of the several regions of the cardiac tube is by no means as clear-cut a phenomenon as it is in the chick and other higher vertebrates. The initial slow beat in the primordial myocardial cone and the subsequent establishing of a more rapid peristaltoid beat sweeping from sinus venosus to aortic outlet is, however, quite in line with conditions seen in the embryos of higher vertebrates.

The meticulous studies of Copenhaver (14, 16) on *Amblystoma* embryos, and Goss (27, 28, 30) on rat embryos, showed that in these representative species of amphibians and mammals the first heart beats appeared in the ventricle, as was the case in the chick. As might be expected there were minor specific differences as to certain details. In *Amblystoma* the first contractions in the majority of the embryos studied appeared in the medial portion of the ventricular myocardium instead of first on the right side as in the chick. In rat embryos the first contractions appeared on the left. The situation as to activity on the right and left of the mid-line is particularly interesting in rat embryos because in their hearts pulsation begins before the paired primordia have fused with each other in the mid-line. It was some two hours following the first discernible contractions on the left before the right half of the ventricular myocardium showed any activity. For a time then each half of the heart beat independently and, surprisingly enough, at a somewhat different rate, that on the left being slightly higher. Dwinnell (19) reports similar observations on the hearts of young rabbit embryos. Under normal circumstances this dual rhythm is a transitory phenomenon, persisting only for the few hours before the right and left primordia fuse with each other to establish a single tubular ventricle. When this occurs unified contraction of the entire ventricular part of the heart is established.

Thus in all the forms as yet critically studied, the ventricle is the first part of the heart to show contractile activity. The first contractions are nonpropagating local twitchings involving only a few cells. In different species there are differences in the precise part of the ventricle to show the first activity but the gradual extension of the

activity from the first areas involved until the entire ventricular myocardium begins regular rhythmic contraction is essentially similar in all forms. The striking peculiarities of this early ventricular beat are its slow rate as compared with that of the heart in older embryos, and its lack of peristaltoid character. These first ventricular pulsations are not adequate to set the blood in circulation.

It is perhaps worthy of comment that the first glycogen in muscular tissue of chick embryos (1) becomes recognizable in cardiac muscle at about the time its contraction begins. Glycogen can be identified in cardiac muscle, in keeping with its earlier assumption of activity, at considerably earlier stages than it can be seen in the developing somatic musculature. It is interesting also that M. R. Lewis (46) found that with special fixation involving the addition of osmic acid to a Zenker-acetic mixture, striated myofibrils could be demonstrated in chick embryos of about 10 somites. One should not conclude from these observations that the initiation of contraction in cardiac muscle is dependent on the presence of glycogen or on the formation of myofibrils. As a matter of fact in these independent observations, both the appearance of glycogen and the differentiation of myofibrils are reported as occurring in embryos about one somite older than those in which Patten and Kramer (60) noted the first evidence of contraction. Goss (29) made a very carefully controlled series of observations using rat embryos which had been kept under continuous observation until the primordial heart started to show its first contractions. When fixed according to the technique of M. R. Lewis, and by other appropriate histological methods, the myocardial elements which had thus been seen to exhibit their first contractions showed no evidence of cross-banding or even of definite myofibrils. Szepsenwol (73) has made similar observations on the explanted hearts of chick embryos. Thus it appears that the earliest contractions occur in cells that are but little specialized morphologically and which still lack the differentiated intracellular structures we are accustomed to think of as characteristic of cardiac muscle. It is, nevertheless, significant that by the time their contractile activity has developed to any degree of efficiency, cross-banded myofibrils can be demonstrated and glycogen becomes recognizable by the standard histochemical tests.

#### SHIFTS IN THE LOCATION OF THE PACE-MAKING CENTER AND EARLY CHANGES IN THE CHARACTER OF THE EMBRYONIC HEART BEAT

One of the most startling and significant facts about the developing embryonic heart is that its myocardium at different cephalo-caudal levels exhibits different inherent rates of contraction. It has been repeatedly demonstrated by fragmentation experiments that the atrial part of the young tubular heart beats faster than the ventricle, and that, later when the sinus venosus is added caudal to the atrium, its rate of contraction is faster than that of the atrium (23, 35, 47, 50, 60, 14). Cohn (12) and Barry (3) have carried out similar studies in greater detail, segregating small fragments taken from a series of locations within each of the main cardiac regions. Their work clearly indicates that the gradient in contraction rate is intracameral as well as intercameral. The gradient in contraction rate can be demonstrated, also, by the less drastic experimental procedure of exerting pressure at the atrio-ventricular region of the cardiac tube sufficient to cause physiological disjunction of the sino-

atrial beat from the ventricular beat (62, 40). This cephalo-caudal gradient in inherent contraction rate is the key to the establishing of a peristaltoid beat of sufficient efficiency to propel the blood through the heart. As might be suspected, the part of the cardiac tube with the highest contraction rate at any given phase of development sets the rate for the entire heart. This is clearly implied by the fact that in transection experiments the sino-atrial part of the heart continues to beat at essentially the rate of the intact heart, whereas any fragment derived from more cephalic regions, when isolated, reverts to the original slower rate of contraction it exhibited before more rapidly pulsating tissue was added to it caudally. The same phenomenon can be more strikingly demonstrated, as was done by Paff (55, 56) by cultivating the parts of transected chick hearts in such close proximity that a bridge of myocardial tissue could grow across from one piece to another. As long as a ventricular fragment remained independent it retained the slow rate it had assumed at the time the heart was transected. When even a slender bridge of tissue grew across connecting the two fragments the more rapidly beating sino-atrial region assumed dominance and caused the ventricle to beat at its own more rapid rate. Even more dramatic were the experiments of Copenhaver (15, 16) in which he grafted the sinus venosus of one species of *Amblystoma* to the ventricular part of the heart of another species with a characteristically different cardiac rate. The transplanted pace-making center took over control of the host ventricle and drove it at the rate of the intact heart of the donor species.

Since the fastest beating part of the myocardium dominates the rhythm of the heart as a whole, and since each new part of the heart that is added behind the first formed cono-ventricular part has a higher intrinsic contraction rate than the part of the heart tube already formed, it follows that there is, during development, a succession of pace-making zones in the tubular embryonic heart. The first beats, as we have seen, appear in the ventricle. This idioventricular pulsation is slow and non-peristaltic in character. As foregut closure progresses caudad and permits more of the paired cardiac primordia to come together and fuse in the mid-line, myocardial tissue exhibiting a higher rate of contraction is added at the caudal end of the slowly beating ventricle. This newly added, faster beating tissue steps up the rate of the heart as a whole and, even more important, it establishes a peristaltoid sweep in the young tubular heart. Since the fastest beating tissue is at the intake end of the heart the waves of contraction there initiated sweep toward the outlet end of the heart, setting up for the first time pulsations of a type which are efficient in propelling the blood. This beat can be characterized as atrio-ventricular. The pace-making zone is, at this time, the atrial myocardium which has just been added caudal to the previously formed ventricle. Moreover, the atrial part of the heart is not formed all at once, but by progressive fusion of the paired primordia. This means that at any given phase of development the most recently added part of the atrium is the pace-maker. Later, as fusion involves still more caudally located parts of the paired cardiac primordia the sinus venosus is added behind the atrium. The myocardium of the sinus has an inherent contraction rate higher than that of the atrium and so in its turn takes over the pace-making function for the heart as a whole. The beat can now be characterized as sino-atrio-ventricular. This is the final major shift in the

location of the pace-making center of the developing heart. With the formation of the sinus venosus caudal to the atrium the basic regional divisions of the heart are all established and the addition of new parts of the cardiac primordia with their progressively higher inherent contraction rates is no longer taking place. There are still minor changes to occur in the concentration and arrangement of the pace-making areas within sinus territory, but they are by no means as radical as the early changes just outlined and they appear in stages of development later than those being covered by this review.

Recently, attention has been directed to the functional importance of the so-called 'cardiac jelly' in the early pumping action of the heart. Davis (17) had applied this designation to the gelatinous material which lies between the endothelial lining of the tubular heart and its outer epimyocardial coat, and had emphasized its significance in giving mechanical cohesion to the two layers of the heart. Patten, Kramer, and Barry (61) in microcinematographic studies of the pumping action of the embryonic heart have shown by superimposed tracings from their moving picture films the manner in which the cardiac jelly is heaped up in local mounds which by their apposition give valvular closure of a hitherto unsuspected type in the embryonic heart. Such mounds appear at two levels, at the constriction between the atrium and the ventricle and in the ventricular conus, at the point where the ventricle discharges into the truncus arteriosus. The time at which these valves close is controlled by the time the peristaltoid sweep of contraction reaches their level in the tubular heart. This means that they act in reciprocal fashion, the valve at atrio-ventricular level closing just after the sweep of contraction through the atrium has fully charged the ventricle with blood, and remaining closed while blood is being forced out through the truncus. At the end of ventricular systole the contraction wave reaches the valvular pads in the conal region which are apposed, thereby checking regurgitation of blood from the arterial stems into the ventricle. While the conal valves are closed the valvular pads at the atrio-ventricular canal are open, thus permitting the ventricle to be charged for its next contraction cycle. It is interesting that this primitive type of valvular action in the tubular embryonic heart appears at regions where the leaf-like atrio-ventricular valves, and the cup-shaped aortic and pulmonary valves are destined to be moulded at much later stages of development. Of possible far-reaching significance, also, is the fact that the mounds when they first appear are shaped from noncellular cardiac jelly, with a subsequent invasion by cells converting the cardiac jelly into a richly cellular connective tissue of the type which has long been called 'endocardial cushion tissue.' It is this readily moulded endocardial cushion tissue which plays such an important rôle in the later development of the cardiac valves and septa, and there arises the intriguing question as to the possible preliminary moulding influence of blood currents on the highly plastic cardiac jelly with the subsequent fixation of these configurations by the more firmly organized cellular tissue which later replaces it.

It is apparent from the material already reviewed that two of the basic factors necessary for efficient propulsion of blood by a tubular pump have been adequately accounted for: (1) The pace-making portion of the heart, shifting in position at different ages but always located at the intake end, starts contraction waves which

sweep through the tubular heart toward its outlet end thus providing an adequate propelling mechanism; 2) the development of valvular endocardial pads at the intake and outlet ends of the ventricle, by minimizing backflow, adds adequate efficiency to the propulsive work of the peristaltoid sweep of contraction. Barry (4) has pointed out that the presence of cardiac jelly between the myocardium and the endocardium is a requisite for a third essential factor, adequate stroke volume. According to his analysis there are certain definite criteria which must be met if a tubular heart is to expel an adequate volume of blood with each beat. The systolic diameter of the heart must be sufficiently reduced to practically obliterate the lumen if the contraction wave is to be effective in forcing blood ahead of itself as it sweeps toward the cardiac outlet. The filling of the heart as well as the amount of blood expelled with each contraction obviously depends upon the diastolic diameter of the myocardial sleeve. Embryonic myocardium, like adult myocardium, can not shorten in systole more than a definite limiting proportion of its diastolic length. In embryonic chick hearts Barry gives this shortening as approximately 20 per cent of the diastolic length. It follows that the myocardial layer must be of relatively large diameter if the stroke volume is to be adequate. If the endocardial lining lay in immediate contact with the myocardial sleeve a functional dilemma would exist. Assuming the lumen were small enough to permit systolic closure by 20 per cent shortening of the myocardium, the stroke volume would be totally inadequate. On the other hand, if a situation were assumed such that the myocardial circumference would be sufficient to allow for adequate stroke volume, there would not be sufficient reduction of the lumen to provide efficient propulsion. The conflict between these two requisites is resolved by the presence of the thick layer of resilient cardiac jelly. This layer transmits the force of contraction of the relatively large myocardial sleeve radially down against the small endothelially lined lumen. Thus the circumference of the contracted myocardium is relatively large, even though the lumen of the heart is squeezed shut, for its radius is increased by the thickness of the layer of cardiac jelly. The increase in the diameter of the myocardial sleeve on diastole under these circumstances will be proportionately large, making it possible for the heart to pump with an adequate stroke volume.

#### ELECTRICAL RECORDINGS FROM EARLY TUBULAR STAGES OF THE EMBRYONIC HEART

In view of the shifts in the location of its pace-making center exhibited by the young embryonic heart at various stages in its development it is obvious that electrocardiographic records obtained during the periods in which these changes are occurring would be of unusual interest, and there have been many attempts to secure such records. The technical problems involved, however, have been varied and troublesome, and until relatively recently the results have been disappointing. As is natural the earliest work in this field dealt with relatively old fetuses which, as might be expected, furnished records quite comparable to those of adults. The first to study the heart of really young embryos by electrocardiographic methods was Wertheim-Salomonsen (77). Using the chick as an experimental animal he succeeded in taking records from embryos as young as 60 hours. He, like other early workers in the field, was greatly handicapped by the lack of amplification methods such as have

since become available, and his records of these early stages showed only slow rises and falls with no clear-cut phases such as would be anticipated from what we know of the character of the heart action at this stage. Only when he worked with chick embryos as old as five to six days did his records show anything like a regular electrocardiographic pattern. Cluzet and Sarvonat (11) and Spadolini and Giorgio (71) encountered the same difficulties, their records from young embryos showing nothing sufficiently consistent to warrant any attempt at interpretation.

That electrocardiograms of adult pattern are obtainable much earlier than was indicated by the foregoing results was shown by Külbs (43), who greatly improved the recording technique and obtained tracings from chick embryos showing the emergence of practically all of the adult characteristics as early as the third day of incubation. In agreement with Külbs' results, Robb (65), in the abstract of her report to the International Physiological Congress, stated that the beginnings of P-waves and of the QRS-T complex were becoming recognizable between 50 and 72 hours.

Further progress in the technique of recording was shown in the work of Lauche and Schmitz (45) and that of Lueg and Höfer (49). Both these papers, however, dealt with explanted hearts or with cultivated heart fragments. Some of such observations, especially those of Lueg and Höfer, Katsunuma and Inada (42), and Szeppenwol and Odoriz (74), on isolated atrial and ventricular portions of the heart are exceedingly interesting, but they need to be evaluated in relation to records of the intact heart acting under more nearly normal conditions.

The work of Hogg, Goss and Cole (39) contains some exceedingly interesting electrical records obtained from cultured ventricular fragments from the heart of 16-day rat embryos. Their observations that diphasic tracings could be obtained by placing the microelectrodes near pulsating centers that were slightly out of phase with each other would seem highly significant. It suggests that one possible factor behind the polyphasic tracings obtained from young embryonic hearts may be an arrangement of the cardiac muscle which sets up a characteristic pattern of areas in a definite sequence of contraction phases. This at least seems like a lead worth further investigation in opposition to the contention of Eyster, Krasno and Hettwer (21) that a polyphasic tracing is characteristic of heart muscle as such.

A description of a carefully worked out technique for recording electrical changes in the embryonic heart was published by Pollack (63). This was followed by a paper in collaboration with Dionne and Schafer (64) on amplification technique, and a later paper by the same group giving some of the results obtained by these methods. The hearts studied by these workers were, however, for the most part rather too old to show the most interesting changes involved in the development of an electrocardiogram of adult configuration.

In a short paper on the influence of digitalis on the embryonic electrocardiogram, Lagen and Sampson (44) state that multiphasic curves are obtainable from chick embryos as early as the 36th hour of incubation. Regrettably, no illustrations of their records were included, so that satisfactory comparison of their interesting findings with other available data is not possible.

Bogue (5) secured clear-cut and convincing records showing the early appearance of polyphasic tracings. Comparing his records with those of earlier workers, it is

apparent that as amplifying techniques have improved the beginnings of the characteristic waves of the adult electrocardiogram have become recognizable in progressively younger embryos.

Taking advantage of the advances in amplification and tissue culture technique Hoff, Kramer, DuBois and Patten (38) obtained electrocardiographic records from a series of embryos in which the degree of heart development was carefully correlated with the tracings secured. The youngest embryo from which they secured satisfactory records was a chick of 15 somites. At this stage, which is reached on the average with about 33 to 36 hours of incubation, the nearly straight tubular heart consists almost entirely of ventricle. The electrical record obtained from it shows none of the deflections characteristic of the adult electrocardiogram, but takes the form of a curve which first drops below, and then rises above, the isoelectric line. This configuration is consistent with the caudocephalic direction of the progress of contraction shown by superimposed tracings of successive frames made by Patten and Kramer (60) from micromoving pictures of the heart action at this stage.

Slightly older embryos (16 to 17 somites, average incubation age 37 to 40 hours) yielded records in which there appears a sharp downward deflection, followed by a rapid return to, or above, the isoelectric line. Because of its characteristic configuration and because correlated morphological studies indicate that the embryonic heart at this stage is practically all ventricle, they interpreted this as representing the QRS complex.

In the next three or four hours of development, fusion of the cardiac primordia progresses caudally, so that the atrial region becomes definitely differentiated and the sinus venosus begins to take shape posterior to the atrium. Records from embryos in this age range showed a downward deflection coming about two twenty-fifths of a second ahead of the QRS complex. This they interpreted as an inverted P-wave.

During the next day of development the ventricular loop is bent backward so that it comes to be in its adult position caudal to the sinoatrial part of the heart. With this shift in relative positions the P-wave appears above the isoelectric line. Thus by the fourth day of development the electrocardiogram has assumed practically its adult configuration.

In connection with the study of the changes in the location of the pace-making center, and the propagation of the contractile impulse as indicated by the electrocardiographic records, the question of nervous control of heart rate naturally arises. The excision and transection experiments reviewed make the primary myogenic character of the pulsation so clear that belaboring of this old issue is uncalled for. Much is still to be learned, however, about the way in which the nervous mechanism secondarily assumes the regulation of the rate of the pulsations initiated within the myocardium. Although the general story of the development of the nerves to the heart is fairly well known (34, 36, 37, 72, 70); much more critical work is needed as to the exact stage of development at which the vagus and cervical sympathetic fibers establish, respectively, their retarding and accelerating action on the heart rate. Such information would be particularly valuable in the case of laboratory animals such as the fowl, the rat, and the rabbit, the embryos of which lend themselves so readily to



experimental procedures. Although work of this type is urgently needed in following through the story of the control of heart rate in later stages, the already available studies clearly indicate that nerve control has not as yet been established in any of the stages here considered. Furthermore, 'conduction tissue' is not at these ages histologically distinguishable from the remainder of the cardiac muscle. There is considerable food for thought in the fact that it is possible to trace the appearance of all the major features of the adult electrocardiographic pattern in embryonic hearts well before they have differentiated a sinoventricular conduction system such as is familiar in the adult heart.

In addition to the work here covered there have been a number of studies on the effect of age differences and temperature changes on heart rate, on the influence of various chemical substances on the character of cardiac pulsation, and, more recently, observations on the effects of hormones or hormone-like substances. Interesting and important as many of these studies are, it did not seem wise to divert attention from the main story of how the heart is established and first starts to function effectively by attempting to cover such work in this review.

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# FACTORS CONTROLLING THE DEVELOPMENT AND PROGRESSION OF DIABETES

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IT IS THE PURPOSE of this review to consider the various metabolic, nutritional and toxic factors which may influence the onset and progression of both experimental and human diabetes.

Recent clinical data, in which nearly three fourths of the 5000 inhabitants of Oxford, Mass., were studied, clearly show that there is a progressive increase in the incidence of human diabetes with age (1). For although the incidence of diabetes was only 0.2 to 0.3 per cent in the 15 to 34-year age group it increased to 4.0 per cent within the 45 to 54-year age group and to 9.7 per cent in patients over 75 years of age.

Since diabetics are usually not born with this disease but rather develop it in the later decades of life, it may be presumed that metabolic and environmental factors influence this onset and progression. One cannot explain the origin of human diabetes on the basis of an hereditary defect alone, inasmuch as such defects would be expected to be present at birth. It may, therefore, be better to consider that the hereditary aspects of diabetes are inherited susceptibilities to these metabolic and environmental factors.

However, before discussing the factors which may influence the onset and progression of diabetes, it is well to consider that there are many types of diabetes which may have different etiological mechanisms.

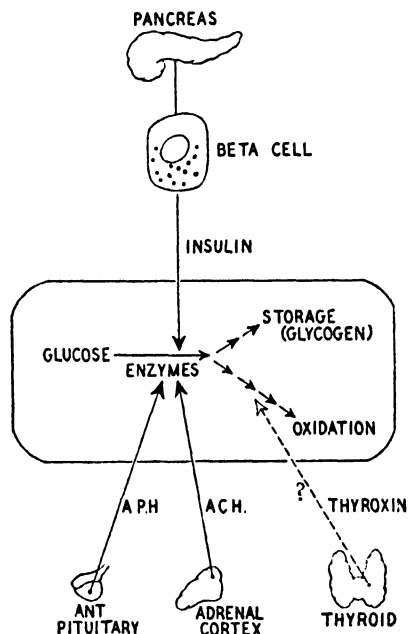
## EXISTENCE OF VARIOUS TYPES OF DIABETES

Diabetes is a disease characterized by hyperglycemia and impaired glucose metabolism. Although the metabolic defect can usually be corrected by the administration of insulin, the diabetic state may be more complex than just a simple insulin deficiency. For, although removal of the pancreas from an experimental animal results in diabetes (2), it has been shown that if either the pituitary gland (3, 4) or the adrenal cortex (5-7) is subsequently removed, the diabetes is ameliorated. Diabetes has also been produced experimentally in normal animals by the administration of large amounts of anterior pituitary (8-10) or adrenal cortical hormone (11). In the case of anterior pituitary hormone injection, a permanent diabetes can be produced which persists even after the cessation of hormone administration (12). Thus, there is an antagonism between the pancreas on the one hand, and the pituitary or the adrenal cortex on the other. If all three of the glands are present and normal, or if the pancreas plus pituitary, or the pancreas plus adrenal cortex are absent, the metabolism simulates normality. However, an imbalance between the pancreas and either the pituitary or adrenal cortex results in a defective metabolism of glucose, and the appearance of diabetes. Figure 1 illustrates some of these endocrine interrelationships.

A glucose molecule upon entering the liver or muscle cell is phosphorylated, and by the subsequent action of a large number of enzymes the resulting glucose-phosphate is either stored as glycogen or metabolized. Although these enzyme reactions can take place in the complete absence of hormones, the hormones do exert a regulatory effect. Thus, if the anterior pituitary or adrenal cortical hormone were to inhibit one or several of these essential enzymes, there would be an interference with the metabolism of glucose, which in turn could result in a hyperglycemia and a failure to store glycogen. If, however, insulin antagonized the inhibitory action of the anterior pituitary or the adrenal cortical hormone upon the enzyme system, then normal metabolic activity would result. This type of endocrine antagonism, which

#### ENDOCRINE FACTORS IN DIABETES

Fig. 1. EFFECT OF VARIOUS ENDOCRINE ORGANS on the metabolism of the liver or muscle cells.



had been known to occur in whole animals (3-7), has recently been reported to occur *in vitro* using an isolated hexokinase enzyme system of muscle (13, 14). Hexokinase is the first of a chain of enzymes involved in glucose metabolism; it is responsible for the initial phosphorylation of glucose and converts it to glucose-6- $\text{PO}_4$ . This phosphorylation is essential for the subsequent oxidation of glucose and its storage as glycogen.

From theoretical considerations one might expect to find several types of diabetes in man.

#### *Pancreatic Diabetes*

A decreased number of the insulin-producing cells (the beta cells of the islets of Langerhans of the pancreas) would result in a decreased insulin output. This would produce an endocrine imbalance and, consequently, diabetes. A decrease in the number of beta cells in the pancreas could result from: 1) a hereditary deficiency,

2) the appearance of toxic factors in the body which are capable of destroying the beta cells already present or 3) the degeneration and exhaustion of overworked beta cells because of excessive demand for insulin. These factors will be discussed in detail later.

It is also possible that in spite of a normal number of beta cells, these cells might be unable to synthesize insulin for metabolic reasons. The amount of insulin precursors, for example, might be a controlling factor in the ability of the beta cells to synthesize insulin; it has been shown that the ability of the body to synthesize protein antibodies is impaired on a low protein diet (15). Since insulin contains 12 per cent cystine (16), the amount of cystine precursors in the diet (cysteine or methionine) may be of more critical importance than the amounts of other amino acids.

In addition to producing insulin, the pancreas is known to produce a second factor (hormone?) which, in contrast to insulin, raises the blood sugar and increases the rate of liver glycogen breakdown (17, 18). Because the distribution of this hyperglycemic factor parallels the distribution of the islet tissue, and because its concentration increases in the pancreas following duct ligation but is not affected following alloxan destruction of the beta cells (18, 19), it has been suggested that this hyperglycemic factor has its origin in the alpha cells. It would be expected that this second pancreatic factor, because of its effect on the blood sugar level, would also play a rôle in the etiology of diabetes.

#### *Pituitary Diabetes Due to Excessive Anterior Pituitary Function*

As we have seen there is an antagonism between the anterior pituitary gland and the pancreas, and an imbalance between the amount of insulin and the amount of anterior pituitary hormone results in diabetes. This imbalance can be produced not only by a deficient pancreas but also might be due to an excessive activity of the anterior pituitary where the pancreas itself may be essentially normal. In two series, totaling almost 1000 acromegalics studied by Borchardt (20) and by Atkinson (21), glycosuria was found in 40 per cent of one series and in 33 per cent of the other. This incidence of glycosuria, therefore, is about twenty-fold the incidence of diabetes in the general population. However, diabetes due to the excessive activity of the pituitary diabetogenic hormone could even be more common than is acromegalia, since the diabetogenic activity of the pituitary might be unduly stimulated without necessarily affecting growth.

#### *Adrenal Cortical Diabetes Due to Excessive Activity of the Adrenal Cortex*

A primary excessive activity of the adrenal cortex could also result in an endocrine imbalance, and might cause diabetes even in the presence of a normal pancreas. Such an imbalance presumably occurs in man (22). Russi and Blumenthal (23) have reported that diabetes occurs five times as frequently in individuals having adrenal cortical adenomas as it does in the general autopsy group. In a dramatic human case of adrenal cortical tumor associated with diabetes, reported by Sprague *et al.* (24), the diabetes completely regressed following the removal of the adrenal tumor. Conn *et al.* have recently produced a temporary diabetes in man by the injection of purified adrenocorticotrophic hormone (25).

### *Thyroid Diabetes*

Diabetes has been produced experimentally by the administration of thyroid hormone to partially pancreatectomized dogs (26). Thyroid feeding produced diabetes only when enough pancreas was removed to lower the pancreatic reserve and yet enough pancreas was left so that the blood sugar remained normal in the absence of thyroid feeding. If the thyroid hormone administration was kept up long enough, the diabetes persisted even after stopping the hormone. Clinically, it is known that the incidence of diabetes in patients with hyperthyroidism is about twice as great as is the incidence of diabetes in the general population (27, 28). It has been observed that the symptoms of mildly diabetic patients are exacerbated following the onset of hyperthyroidism, and that treatment of the hyperthyroidism improves the diabetes (29).

### *Liver or Muscle Diabetes*

Reference to figure 1 shows that a decrease in enzyme activity can result not only from an endocrine imbalance but also if the amount of enzymes themselves within the liver or muscle cells is decreased. A decrease in enzyme concentration would also impair the oxidation and storage of glucose. Thus, in spite of a normal pancreas, normal pituitary, adrenal cortex and thyroid gland, a primary defect in the amount of an enzyme, such as hexokinase, could result in a decreased phosphorylation of glucose and, hence, a decreased oxidation and storage of glycogen, i.e. diabetes. Enzyme deficiencies are known to occur in man, as, for example, in glycogen storage diseases (30), phenylketonia (31, 32) etc. In animals, certain types of toxic liver disease are characterized by a decreased glucose tolerance and impaired glycogen storage (33, 34). In man, it has been reported that the 'diabetic-like state' associated with certain cases of biliary disease is improved upon treatment of the liver disease (35, 36).

### *Diabetes Due to an Abnormal Insulin Sensitivity or Destruction*

Himsworth (37) has shown that human diabetics may be subdivided into insulin sensitive and insensitive types. The latter show relatively little change in their blood sugar following a reasonable insulin dose. In experimental animals it has been noted that the diabetes occurring during the injection of anterior pituitary (38) and adrenal cortical hormones (39) is of an insulin insensitive type.

Although little is known about the excretion, destruction or inactivation of insulin within the body, it is evident that any factor which would increase the rate of removal of insulin from the body, whether it be inactivation, destruction or excretion, would tend to produce a diabetic-like state. It is known that insulin may be inactivated *in vitro* with glutathione or cysteine (40); whether such inactivation also takes place within the cells of the body by the glutathione present is not known. Certainly much more information is needed about the fate of insulin in the human body.

Unfortunately, it has not yet been possible to determine the etiological mechanism for every given case of diabetes, and we are therefore unable to determine the relative frequency of the various primary types of human diabetes.



## ROLE OF THE PANCREAS IN THE ETIOLOGY OF HUMAN DIABETES

*Pathology of Diabetes vs. Beta Cell Granule Content*

Because of the fundamental studies on the pancreas by Bensley and Lane it is now apparent that there are several distinct types of cells in the islets of Langerhans of the pancreas (41). It is the beta cells in the islets which are primarily involved in pancreatic diabetes (42-45). When beta cells degenerate as a consequence of a wide variety of experimental procedures, they undergo a sequence of pathological changes which have many similar features in spite of being produced by different etiological mechanisms. These changes consist of a loss of the specific beta cell granulations, the appearance of vacuoles in the cytoplasm (hydropic degeneration), nuclear pyknosis, cytoplasmic shrinkage, and finally the complete disappearance of the beta cells. These reactions are usually characterized by a lack of inflammatory reaction in the islets. The type of reaction observed depends not only on the severity of the toxic factor employed but also upon the time of observation for the beta cells may pass through several successive stages of degeneration. Thus in the case of alloxan diabetes, the beta cells rapidly pass through successive stages and have almost completely disappeared in two to three days (45-47). In the case of partial pancreatectomy (44) or pituitary diabetes (48, 49) the stages persist for much longer times and frequently various beta cells within a given pancreas show different types of reaction. Although some of these changes are easily observed in tissues prepared by standard histological methods with routine pathological stains such as hematoxylin-eosin, other changes, such as degranulation or disappearance of beta cells, necessitate the use of special cytological methods. It has not been fully appreciated that a pancreas which contains practically no beta cells, such as is the case in alloxan diabetic rats, examined 5 to 6 days after alloxan injection, may appear essentially normal when studied by the ordinary histological stains; whereas, if this same pancreas is studied by means of the specific granule stains, it is found to contain practically no beta cells (50). In alloxan diabetes in man it is similarly reported that routine hematoxylin-eosin stains revealed slight changes in the beta cells; whereas, specific granule stains revealed profound changes (51).

It is not surprising, therefore, that the early reports on the pathology of the islets of Langerhans in human diabetes are confused primarily because these studies were made without reference to the specific cell types occurring within the islets. Although some investigators such as Wechselbaum (52) and Allen (44) have stressed the importance of hydropic degeneration in the islets of diabetics, others have emphasized the occurrence of hyalinization and fibrosis (53-55). Occasionally nuclear pyknosis has been observed (55, 56). This hyalinization or fibrosis which is found occasionally in non-diabetics as well may not be the cause of the diabetes, but rather it may be an accompaniment similar to other complications of diabetes such as arteriosclerosis (55) and intercapillary glomerulosclerosis (57). Although these lesions might secondarily contribute to beta cell degeneration, they need not always be present. The fact that the observed incidence of hyalinization increases with age and the duration of the diabetes (55) supports the thesis that it is a secondary complication. In view of the fact that hydropic degeneration of the beta cells

which is observed in some types of experimental diabetes can be controlled by treatment (42, 43, 58, 59), one would further expect that this lesion would be a less common pathological finding in human diabetes in the present post-insulin era.

Of primary diagnostic importance, therefore, is the evaluation of the number and state of the beta cells in the pancreas of diabetics, and for this evaluation, special fixation and staining methods are required. Recent human studies by Gomori (60) and by Bell (61), using special stains for differentiating alpha and beta cells, have shown that a large percentage of diabetics do have significant changes in their beta cells. Gomori did not attempt to evaluate the absolute number of beta cells present, but rather estimated the ratio of beta to alpha cells. He found that the pancreatic islets of 4 out of 7 diabetics showed a significant decrease in this ratio. Bell, in a study of 30 diabetics, reported that the islets of 11 showed no beta granules, 10 showed only a small number of beta granules, 5 showed a moderate decrease, and only 4 of the 30 were normal. In contrast, all of the 50 non-diabetics studied showed a normal content of beta granules. Thus, 70 per cent of all diabetics studied by Bell had a complete absence or a marked decrease in the beta cell granule content. The fact that 13 per cent of the diabetics studied did not show any change in the number of their beta granules is probably explained by the fact that these cases of diabetes were due to other causative factors. For example, a primary liver diabetes or a pituitary diabetes in its early stages might not be expected to show morphological changes in the pancreas.

#### *Insulin Content of Pancreas in Human Diabetes*

The average insulin content of the pancreas of 18 diabetics was found to be only 25 per cent of that found in the normal (62). One moderately severe diabetic who had been controlled with insulin for many years had a normal pancreatic insulin content; whereas, another patient that died in diabetic coma had a very low insulin content (less than 4 per cent of normal). These figures further substantiate the view that not all diabetes is pancreatic in origin, but they do suggest that a large percentage of diabetics do show a significant reduction in the insulin content of the pancreas.

#### *Insulin Requirement of Diabetic vs. Total Depancreatized Man*

It has been found that the insulin requirement of a total depancreatized man is less than that of many diabetics (63, 64). Brunschwig *et al.* (65) removed the entire pancreas in a human diabetic patient and found that the insulin requirement decreased following the operation. For these reasons serious doubts have arisen as to the rôle of the pancreas in the etiology of human diabetes (65, 66). In an analogous study by Thorngood and Zimmerman (67), the glucose excretion (without insulin) and the insulin requirement were carefully measured in alloxan diabetic dogs. The pancreas was removed and the insulin requirement and glucose output were re-determined. It was found that removal of the pancreas in the alloxan diabetic dog decreased both the glucose excretion and the insulin requirement. In light of the hyperglycemic factor which has recently been found in the pancreas (17-19) these results can be best explained (67) by the presence of an accessory hyperglycemic factor

(alpha cell hormone?) in the alloxan diabetic dog which is absent in the depancreatized dog. These results may also explain the discrepancy between the insulin requirement of many human diabetics and that of the total depancreatized man. They suggest the need for further evaluation of the rôle of the pancreatic hyperglycemic factor in the etiology of human diabetes.

#### FACTORS CONTROLLING DEVELOPMENT AND PROGRESSION OF DIABETES

In view of the frequent beta cell involvement in human diabetics and because of the progressive increased incidence of diabetes with age, it seems likely that in many of the individuals who develop diabetes there is a progressive degeneration and loss of beta cell function. However, it must be emphasized that diabetes due to other causes may also occur in which the pancreas is not involved and then the beta cells would be entirely normal. The fundamental problem is: Why does beta cell degeneration take place and what are the metabolic and nutritional factors which influence it.

#### *Toxic Substances Capable of Destroying Beta Cells*

It has recently been shown by Dunn and co-workers (45) that alloxan selectively destroyed the beta cells of the pancreas and thereby offered a new tool for the production of experimental diabetes (68, 46, 47). Alloxan produces necrosis of the beta cells without affecting the alpha cells. In the acute stages following alloxan injection, degranulation, nuclear pyknosis and necrosis are observed. In the chronic stage (weeks or months after alloxan injection) the beta cells have disappeared, and the islets are composed primarily of alpha cells and a few non-granular cells. In the late stages hydropic degeneration of the few beta cells still remaining has also been observed (69). It should be reemphasized that on histological examination of the pancreas of such a chronic alloxan diabetic rat using routine staining methods, the islets may appear normal (50). It has been found that the administration of large doses of glutathione will protect the beta cells and prevent degeneration (70, 71). In a recent preliminary paper by Griffiths (72a), it has been claimed that the injection of large doses of uric acid produced a transitory diabetes in rabbits.

In subsequent studies (72b) it was found that a permanent diabetes was produced when two injections of uric acid (1 gm/kg.) were given. Histological studies of the pancreas indicate that the beta cells of uric-diabetic rabbits showed cytological changes similar to those observed following alloxan injection (72b).

#### *Exhaustion and Degeneration of Beta Cells Due to Excessive Demand for Insulin*

When part of the pancreas is removed, the remaining pancreatic remnant is called upon to substitute for the whole pancreas and becomes overworked. When 80 per cent of the pancreas was removed from a dog or cat and diabetes did not develop, the remaining beta cells enlarged and showed degranulation (42, 43). By contrast, when more than 80 per cent of the pancreas was removed and diabetes developed, the beta cells showed hydropic degeneration, the cell outline became irregular and the nuclei became shrunken and pyknotic. The alpha cells appeared unchanged. The degree of beta cell change in the pancreatic remnant closely paralleled the

severity of diabetes in the animal and if the work of the remaining beta cells was lessened by treatment of the diabetes, these degenerative changes in the beta cells could be prevented. Thus, when the beta cells were called upon to function excessively, they first became degranulated and then they became 'exhausted', degenerated, and tended to disappear.

*Secondary Degenerative Changes in Beta Cells Which Develop as a Consequence of a Primary Endocrine Imbalance.*

In the section on CAUSES OF DIABETES it was pointed out that primary over-activity of the anterior pituitary could result in a defective metabolism of glucose by the liver cells and thus result in hyperglycemia and glycosuria. Although initially the beta cells of the pancreas may be normal, repeated anterior pituitary injections do result in secondary degenerative changes in the beta cells (73, 74). Initially these changes consist of degranulation and hydropic degeneration of the beta cells. The alpha cells are unaffected. Later when the diabetes has become permanent the beta cells not only show degranulation and hydropic degeneration but many of them have disappeared. Thus, although the diabetes started as a primary pituitary diabetes, it developed into a pancreatic diabetes as well. A similar phenomenon has been shown to occur in thyroid diabetes (26), and may also occur in adrenal cortical diabetes.

*Glucose as a Factor in Causing Degeneration of Beta Cells*

Woerner has shown that large doses of glucose, injected intravenously into guinea pigs, produce degranulation and hydropic degeneration of the beta cells (75). In experimental diabetes, produced by the injection of anterior pituitary hormone, it has been observed that the injection of phlorhizin, which lowers the renal threshold causing a urinary excretion of the sugar, cured the diabetes and reversed the degenerative changes in the beta cells (59). Lukens *et al.* believe that the hyperglycemia *per se*, resulting from the injection of anterior pituitary hormone, is responsible for the progressive degeneration of the beta cells (59). More recently Dohan and Lukens (76) have produced permanent diabetes in partially pancreatectomized cats and even in one normal cat by the injection of massive doses of glucose.

*Diet and Development of Diabetes*

Interest in the relation of diet to the development of diabetes probably dates back to the discovery of glycosuria. Allen in 1913 (43) carefully evaluated the rôle of diet in the partially pancreatectomized dog and found that when mildly diabetic dogs were fed a meat diet they put out little glucose in their urine whereas when fed a high bread diet they excreted large amounts of sugar. More recent studies have shown that the time of onset and the progression of diabetes is markedly influenced by diet.

**EFFECT OF DIET ON PROGRESSION OF DIABETES IN PARTIALLY PANCREATECTOMIZED RAT.** Foglia has carefully evaluated the progression of symptoms following sub-total pancreatectomy in the rat (77). After the removal of 80 to 95 per cent of the pancreas, there is an initial period of obesity with no manifest signs of diabetes.

This stage is then followed by a period of manifest diabetes with alimentary hyperglycemia appearing first, which is in turn followed by fasting hyperglycemia and glycosuria, and finally by a period of ketosis and death. The rate of progression through these different phases of diabetes was found to depend upon the amount of the pancreas removed. This progression of diabetes in sub-total pancreatectomized rats parallels in many ways the development of human diabetes; for, in man, diabetes is also frequently preceded by a period of obesity, which may then progress to a stage of alimentary hyperglycemia, and finally to manifest diabetes.

Houssay and Martinez (78, 79) have recently studied the effect of various diets on the rate of development of diabetes in 95 per cent pancreatectomized rats. The diets contained varying amounts of wheat flour, corn flour, casein and lard, and 5 per cent yeast, 5 per cent cod liver oil and 1 per cent salts. The percentage of the total calories which was consumed in the form of carbohydrate, fat and protein has been calculated from their data (78, 79) and is tabulated in table 1.

TABLE 1. DIETS USED BY HOUSSAY AND MARTINEZ (78, 79)

TYPE OF DIET	CALORIES DERIVED FROM		
	Fat	Carbohydrate	Protein
	%	%	%
High fat	63	20	17
High carbohydrate	12	59	29
High protein	12	37	51
Low protein	13	68	19

*Effect of high carbohydrate, high fat, and high protein diets.* Sixteen to eighteen rats were placed on each of the following diets: high carbohydrate, high fat and high protein. The animals were carefully followed for 8 months and the incidence of diabetes noted. At two months, 56 per cent of the rats on the high protein diet developed diabetes, as contrasted with 78 per cent on the high carbohydrate diet, and 100 per cent of the rats on the high fat diet. Looking at their data in another way, it is noted that within 2 months all of the rats on a high fat diet developed diabetes; on the high carbohydrate diet all of the rats developed diabetes in 7 months; whereas, even after 8 months only 82 per cent of the rats on the high protein diet developed diabetes.

The rats on the high fat diet gained more weight than did those on the high protein diet; whereas, those on the high carbohydrate diet gained the least. The more rapid rate of development of diabetes on a high fat diet is not simply the result of the increased caloric intake, even though it will be shown presently that a high caloric intake also accelerates the rate of development of diabetes. When 95 per cent pancreatectomized rats were fed a fixed caloric intake, those on a high fat diet still developed diabetes more rapidly than did those on a high carbohydrate diet.

When comparing the rate of development of diabetes on the high carbohydrate versus the high protein diet, one cannot differentiate between the effects of high carbohydrate and low protein in the diets. Since the protein percentage of the diet is

indirectly increased as the carbohydrate content of the diet is decreased, there are two simultaneous variables. Therefore, the more rapid rate of development of diabetes on the high carbohydrate, as compared to the high protein diet, may be due either to a toxic effect of the high carbohydrate diet or to the lack of a protective factor which may be present in the high protein diet.

*Effect of amount of calories consumed.* In a second series of experiments all rats were fed the high carbohydrate diet, but the amount of food eaten was varied. One group of animals was fed three times daily and given all the food they could eat; a second group (the normal food intake group) was fed only once a day; whereas the third group of animals was also fed once a day, but was given only 80 per cent of the food consumed by the normal rats. Thirteen to eighteen rats were placed in each group. After 2 months of this dietary regime all of the overfed animals had developed diabetes; whereas only 78 per cent of the normally fed and only 42 per cent of the underfed had developed diabetes. Looking at this data in another way, it is noted that all of the overfed rats developed diabetes within 2 months; all of the normally fed rats developed diabetes within 7 months; and even after 8 months only 43 per cent of the underfed rats had developed diabetes. It does not seem likely that the more rapid progression of diabetes observed in the normally fed as compared with the underfed rats is related solely to the increased amount of fat consumed with increasing amounts of the diet. Since the high carbohydrate diet contains only 5 per cent fat, which accounted for only 12 per cent of the calories consumed, the absolute increase in fat intake is very small indeed. On the other hand, when the diet contains 54 per cent carbohydrate an increase in total dietary intake of only 20 per cent would cause a much larger absolute increase in carbohydrate intake. These experiments, therefore, suggest that it is either the increased amount of carbohydrate or protein consumed which results in the more rapid development of diabetes. Since it was previously concluded that the more rapid progression of diabetes on a high carbohydrate diet, as compared to a high protein diet, was due either to a toxic effect of the carbohydrate or to a protective factor present in the high protein diet, the present results observed with varying the amount of diet fed suggest that it is the carbohydrate in the diet which exerts an unfavorable influence on the progression of diabetes, since the incidence of diabetes increases despite the increasing protein intake.

*Effect of single vs. multiple feedings.* In a third set of experiments (78, 79) 95 per cent pancreatectomized rats were placed on the high carbohydrate diet, and the same amount of diet was fed to each group. However, in one case the food was fed once a day; in the second, the food was divided into three equal portions and fed three times a day. Thirteen or fourteen animals were placed in each group and at the end of 3 months there was not any significant difference between the two groups. After 5 months, however, 50 per cent of the animals fed once a day developed diabetes as compared with 23 per cent of the animals fed the same total diet in 3 divided daily doses. The weight and growth curves for the two groups of animals were nearly identical. Unfortunately, since the number of rats developing diabetes was small, this experiment needs to be extended to include a much larger group of

animals, for statistical calculations<sup>1</sup> indicate that the differences observed could have happened by chance one out of six times. Houssay and Martinez suggest that feeding the same diet once a day may have resulted in a longer and possibly greater hyperglycemia than was the case when the same diet was divided into three feedings, and they propose that this hyperglycemia may be a contributing factor to the more rapid progression of diabetes.

**EFFECT OF DIETS ON SUSCEPTIBILITY OF RATS TO ALLOXAN.** Houssay and Martinez also studied the effect of various diets on the sensitivity of rats to alloxan (78). The three diets previously mentioned and a fourth—a low protein diet—were used (table 1). Rats were placed on the various diets for one month and then injected with alloxan intraperitoneally in doses of 160 mg/kg. They reported the mortality following this dose of alloxan and stated that in each case the animals that died had a high blood sugar. Interpretation of this study is a little more difficult because it combines a number of factors. For example, the previous diet may influence the effectiveness of alloxan in destroying the beta cells of the pancreas. Once the beta cells are destroyed, however, the diet may also influence the severity of the diabetes and this may in turn hasten death. No data is given as to the percentage of injected animals which developed diabetes, but which did not die of it within the first week. In spite of these difficulties, their findings are most significant.

The sensitivity of animals to alloxan seems to be affected by the amount of protein in the diet. In animals on a low protein diet, (19 per cent of the calories derived from protein and 68 per cent from carbohydrate), 90 per cent of the rats died of diabetes following the alloxan injection. On the other hand, on the high carbohydrate diet (29 per cent of calories from protein and 59 per cent from carbohydrate), the mortality rate was only 40 per cent. On the high protein diet (51 per cent of calories from protein and 37 per cent from carbohydrate), the mortality rate was 33 per cent. Comparing the high protein diet with the high carbohydrate diet, there is little difference in terms of the susceptibility of rats to alloxan. However, it should be pointed out that the high carbohydrate diet still had a large amount of protein. In the above three diets the only fat in the diet was that in the 5 per cent cod liver oil fed.

Feeding rats with a high fat diet, containing either lard or ox fat (63 per cent of the calories derived from fat) increased the mortality due to alloxan (90–100 per cent mortality), as compared with either the high carbohydrate or the high protein diet. On the other hand, fats such as olive oil, butter or oleomargarine fed in essentially the same amounts did not increase the mortality (21–40 per cent) from diabetes; furthermore, it appears as if coconut oil and corn oil are protective (0–13 per cent mortality) when compared to even the high carbohydrate and high protein diets. When 10 per cent coconut oil was added to the high lard diet it decreased the diabetic mortality following alloxan (20 per cent diabetes mortality as compared to 100 per cent for the lard alone). Thus lard and ox fat appear to sensitize rats to the development of alloxan diabetes; whereas, coconut oil appears to exert a protective effect.

Methionine was found to afford some protection against the high lard diet, for when the high lard diet was supplemented by 200 mg/kg. of methionine per day, the

<sup>1</sup> By Dr. G. F. Badger, Department of Preventive Medicine, Western Reserve University, Cleveland, Ohio.

mortality rate was only 30 per cent, as compared to the diabetic mortality of 100 per cent on the unsupplemented high lard diet. The mortality rate due to the high lard diet plus methionine is about the same as that observed on the high protein diet. Since choline did not protect against the high lard diet, these results suggest that the protective effect of methionine is not due to its capacity to donate labile methyl groups, as choline also shares this property, but rather to the ability of methionine to donate its sulphur groups. The fact that sulfanilamide also does not protect against a high lard diet suggests that the sulphur must be in an appropriate form. This protection afforded by methionine is similar to the acute sulfhydryl protection against alloxan diabetes first reported by Lazarow (70).

**RÔLE OF DIET IN DEVELOPMENT OF DIABETES IN MAN.** In a human study Himsworth (80) correlated the diabetic death rate with the amount of fat consumed by classes and races of individuals. He reports that the diabetic mortality rate bears a direct relationship to the estimated amounts of fat eaten by the various groups. The increased incidence of diabetes, which has occurred in the countries of western civilization during the last 30 years, has occurred concurrently with the change in dietary preference in which a progressively greater proportion of fat and a smaller proportion of carbohydrate have been chosen. The proportion of protein and the caloric value of the diet is said to have remained unaltered. The fall in diabetes mortality which occurred during World War I was also thought to be related to the reduction in the amount of fat in the diet. This reduction of diabetes mortality was accompanied by only a slight reduction of caloric intake in most countries except for Germany. The higher mortality due to diabetes in urban as compared to rural populations is also correlated with the smaller proportion of carbohydrate and the greater amounts of fat consumed by the city population. Immigrants, who go to a new country and gradually acquire the dietetic preference prevalent in the new land, show an increase in the diabetic incidence rate if the newly acquired diet also contains a greater proportion of fat to carbohydrate than did the diet of the native land. A rise of economic position is associated with a corresponding rise in the diabetic incidence rate, which Himsworth feels is also associated with a change in dietary habits in that a smaller proportion of carbohydrates and a greater amount of fat is taken.

Human dietary studies are certainly much more difficult to evaluate than are those of animals. The more effective diagnosis of diabetes may, in part at least, explain the increased incidence of diabetes during the past 30 years and may even enter into comparisons of urban and rural populations. A change in economic status may also be associated with a change in total calories consumed, as well as in the proportions of fat and carbohydrate. It is of considerable interest, however, that in spite of these limitations, the observations reported by Himsworth on man are fairly similar to the animal experiments reported by Houssay and Martinez (78, 79) in which a high fat diet (lard or ox fat) increased the incidence rate of diabetes in partially pancreatectomized rats. However, one cannot conclude that high carbohydrate does not have an effect in man. If the toxic effect of a high fat diet on the development of diabetes is much more marked than is the case of the high carbohydrate diet, then the effect of increasing the carbohydrate may be obscured by the



more pronounced effect of the concomitant decrease in the fat intake. This actually seems to be the case in the animal experiments. One cannot conclude, therefore, from the studies of Himsworth that a high carbohydrate intake does not influence the rate of development of diabetes in man without devising an experiment to specifically test this hypothesis, since as the percentage of carbohydrate fed is increased, the fat is consequently decreased and one cannot separate these effects.

*Other Metabolic Factors Which Influence Development of Diabetes*

*Acetoacetic acid.* It has been found that the daily repeated injections of acetoacetic acid and beta-hydroxy-butyric acid into guinea pigs increased the insulin content of the pancreas for the first 2 to 4 weeks and later decreased it to 50 percent of normal after 70 days (81). It has been claimed that the repeated injection of acetoacetic acid, an intermediary product in fat metabolism, produced hyperglycemia and abnormal glucose tolerance in rabbits (82), and that insulin itself is inactivated both *in vitro* and *in vivo* by acetoacetic acid (83). Although other investigators (84) failed to observe an abnormal glucose tolerance in rats injected with repeated doses of acetoacetic acid, they did observe a significant drop in blood sugar and suggested that acetoacetic acid may stimulate the secretion of insulin. Since variation in species response to the injection of anterior pituitary hormone accounts for a reduced insulin content of the dog pancreas, and an increased insulin content in the rat pancreas (85), these apparently conflicting observations on acetoacetic acid must be clarified since they were carried out on different species. These effects of injected acetoacetic acid may account for the increased fasting blood sugar levels which are observed when men are placed on a high fat diet (86, 87). For example, epileptic patients fed 260 to 580 gm. of fat and 4 gm. of carbohydrate a day showed an increase in the fasting blood sugar from an initial value of 135 mg/100 cc. to a value of 357 mg/100 cc. after 7 weeks (86).

*Vitamin C deficiency.* Guinea pigs placed on a scorbutic diet for 21 days showed a decreased glucose tolerance as compared to controls which were pair fed the same diet supplemented with ascorbic acid (88, 89a). Not only was the insulin content of the pancreas reduced (89a) but the beta cells of the pancreas showed definite signs of degeneration (89b).

MECHANISM OF BETA CELL DEGENERATION AND MODE OF ACTION OF DIETARY FACTORS

Because of the progressive beta cell degeneration which follows sub-total pancreatectomy and anterior pituitary hormone administration the concept of 'overwork exhaustion' or 'pancreatic strain' has been proposed (42, 43, 59, 85). Appealing as these terms are, they give no insight into the mechanism of beta cell degeneration and this must, therefore, still be explained in more precise biochemical terms.

Since there is ample evidence that beta cell degeneration does take place both in the experimental animal and in the human subject it would seem wise to consider beta cell degeneration from two standpoints: the peculiarities of the beta cell which may make them more susceptible to degeneration and toxic factors capable of destroying them. Since the metabolism of the insulin-secreting tissue is intimately tied up with the susceptibility of the beta cells to degeneration, the peculiarities of beta cell metabolism will be considered first.

### *Rôle of Cysteine in Insulin Synthesis*

Inasmuch as insulin contains a very large percentage of cystine (16), a much larger one than most other proteins in the body, the availability of the precursors of cystine (methionine and cysteine) may be critical to insulin synthesis. Since the body cannot completely synthesize cysteine unless methionine is present in the diet (90), these amino acids must be available in adequate amounts. They must reach the beta cells and there, under the influence of enzymes, be incorporated into the cystine portion of the insulin molecule (fig. 2). Low protein diets would tend to accentuate deficiencies of cysteine plus methionine and might, therefore, decrease the ability of the beta cells to synthesize insulin, and thus contribute to the diabetic state.

### *Rôle of Glutathione in Physiological State of Beta Cells*

In addition to being a constituent of protein, cysteine is a constituent of glutathione. It has been shown that many enzymes of the body contain sulfhydryl groups, which are essential for their activity (91-94). If these sulfhydryl groups are inactivated, the enzymes themselves become inactive. In many cases it can be shown that the enzymes may again be reactivated by the addition of glutathione. Therefore, it is believed that glutathione, the naturally occurring sulfhydryl tripeptide, serves to protect the sulfhydryl groups of enzymes and thus maintain them in an active state. Although little is known about the enzymes of the beta cells or those involved in insulin synthesis, it is very probable that many of the enzymes in the beta cells (i.e. succinic dehydrogenase (91), etc.), do require active sulfhydryl groups. The amount of glutathione present in the beta cells may, therefore, be expected to affect their physiological susceptibility to degeneration and their susceptibility to toxic substances.

In 1945 Lazarow first reported that the injection of large doses of glutathione immediately preceding a diabetogenic dose of alloxan completely protected rats from diabetes (95, 70). Although many other substances have been reported to protect against alloxan diabetes (95, 96, 97, 70, 98, 99, 100), glutathione is of particular interest because it is a natural constituent of cells. The concentration of glutathione in the various organs ranges from about 30 to 200 mg. of glutathione per 100 gm. of tissue. It is of considerable interest to note that the dose of glutathione which was found to completely protect rats against a diabetogenic dose of alloxan (40 mg/kg.) was found to be 2mm/kg. or 62 mg. of glutathione per 100 gm. of body weight which is within the normal physiological range. It is, therefore, evident that the glutathione which is normally present in the body can serve as a natural protective factor against alloxan diabetes.

### *Mechanism of Alloxan Action*

Glutathione reacts with alloxan to reduce it to dialuric acid (114, 101, 102) which is not diabetogenic (101). The sulfhydryl group of glutathione further reacts with alloxan to form a new compound which has an absorption spectra maxima at 305 m $\mu$  and this compound is presumed to be an addition product (101, 102). It is not sur-

prising, therefore, that when alloxan is injected into the body there is a fall in the glutathione content of the tissues (103-105).

Since the blood is the initial site of reaction with alloxan, the blood glutathione content may fall to near zero values (104); whereas, the tissues, being secondary sites of reaction, may show a lesser fall (103, 105).

Alloxan also reacts with the sulfhydryl groups of protein in a manner similar to its reaction with glutathione. The sulfhydryl groups are not only oxidized to the disulfide form (106), but they also react with alloxan and give rise to a new absorption spectra compound which has a maximum at 305  $m\mu$  (101, 102). Since alloxan reacts with the sulfhydryl groups of proteins, it would be expected that sulfhydryl enzymes would be inactivated by alloxan and this has been found to be the case (91, 92). Because many of the essential enzymes of the cell require sulfhydryl groups for their activity it has been suggested (70) that the diabetogenic action of alloxan may be due to its ability to react with the essential sulfhydryl groups of enzymes and thus produce cell death. However, if alloxan produces diabetes by inactivation of essential sulfhydryl enzymes, one may ask why other sulfhydryl inhibitors do not produce diabetes.

Since the various known sulfhydryl inhibitors are not equally effective in their action on crystalline sulfhydryl enzymes, one might not expect them to be equally effective in producing diabetes. Thus under certain conditions crystalline yeast hexokinase is inhibited 15 per cent by  $1 \times 10^{-3}$  molar iodosobenzoate, 24 per cent by the same concentration of iodoacetamide and 99 per cent by one tenth the concentration of para chloro-mercury benzoate (107).

Another factor which influences the effect of various sulfhydryl poisons is the local concentration of the inhibitor. In the case of arsenic and mercury these compounds are excreted by the intestine and the kidney (108) and the local concentration in these organs may reach a toxic level whereas the concentration in other parts of the body (i.e. the beta cells) may be subthreshold. Thus although an animal may die of gastrointestinal hemorrhage or uremia, beta cell damage would not be expected to take place unless the beta cells too could concentrate the drug. When, however, very large doses of arsenic or mercury are given to an animal, selective symptoms may not have time to develop and acute death ensues. Acute death also occurs following the administration of large doses of alloxan; in fact the symptoms following the administration of large doses of alloxan have been compared to those in acute arsenic poisoning (109).

There are also several types of reactions that occur between inhibitors and sulfhydryl groups. Some inhibitors involve an oxidation to the disulfide or to a more completely oxidized form; some, such as arsenic and mercury, involve the formation of a reversible addition product; whereas, others may involve the formation of irreversible combinations (110, 111). Glutathione can reactivate sulfhydryl enzymes inactivated by oxidation to the disulfide form, and can remove arsenic and mercury from combinations (112); however, it cannot reduce sulfhydryl groups oxidized past the disulfide stage, nor can it reverse the effect of some of the alkylating agents. It is of particular interest to note that the inhibition of succinic dehydrogenase by alloxan is reported to be only partly reversed by glutathione (91) indicating that two

types of inactivation have taken place. It might be expected that a compound which inactivates sulfhydryl groups irreversibly, and this may prove to be the case with alloxan, would be a more effective diabetogenic agent than would a reversible inhibitor. And it has been found that whereas sulfhydryl compounds protect against diabetes when they are given prior to the alloxan glutathione, cysteine or BAL do not modify the course of the diabetes when they are given five minutes after the alloxan (70, 99).

The possibility also exists that alloxan may react with other groups of protein as well as with sulfhydryl groups. Although it has been shown that the reaction between alloxan and glutathione or protein must take place through the sulfhydryl group since oxidized glutathione does not give compound '305', it is likely that additional linkages are also involved. For cysteine does not react with alloxan to form '305' in spite of the fact that it has a free sulfhydryl group. It would seem likely, therefore, that additional amino acid groupings may also be reacting with alloxan (102). If alloxan reacts with other groups of protein in addition to the sulfhydryl groups, or if it oxidizes them further than the disulfide form, alloxan might produce a more effective enzyme inactivation than would be the case with other sulfhydryl inactivators.

However, if beta cell death is a result of sulfhydryl enzyme inactivation, why then does alloxan selectively kill the beta cells, for certainly essential sulfhydryl enzymes are present in all cells? It is most unlikely that appreciable concentration of alloxan could take place in the beta cells since alloxan is so rapidly destroyed in the body (104, 113-115). However, alloxan in large doses kills other cells (liver and kidney, etc.) (68, 47, 71) in addition to beta cells, and in very large doses (560 mg/K) it is acutely toxic to whole animals (109). Since glutathione is a naturally occurring protective compound, it was suggested that a low beta cell glutathione content would explain the selectivity of alloxan for the beta cells (70), and, as will be discussed presently, there are theoretical reasons which suggest that the synthesis of insulin may deplete the beta cell glutathione (116).

The analyses of the glutathione content of the pancreas give no indication as to the glutathione content of the beta cells since these latter cells constitute but a small fraction (about  $\frac{1}{2}$  per cent) of the total weight of the pancreas. However, it should be pointed out that glutathione is primarily intracellular and it does not readily leave the cell. All the glutathione contained in the blood is present in the red cell and it cannot be removed by washing blood corpuscles in saline (117a), similarly the glutathione contained in the liver is only slowly removed on perfusion (117b). Therefore, the glutathione in the various tissues of the body is not in rapid equilibrium. Furthermore, when alloxan is injected into rabbits, there is an immediate drop in the blood glutathione to almost near zero values; however, even after 5 hours the blood glutathione has not yet returned to its original level (104) in spite of the fact that there are large amounts of glutathione in other tissues.

The sulfhydryl theory of alloxan action has been criticized on the basis that following the injection of a diabetogenic dose of alloxan, although the blood glutathione had fallen to near zero levels, the pancreas still contained practically normal amounts of glutathione (105). It was suggested, therefore, that the glutathione of

the ascinar tissue would be available to reduce any disulfide groups which had been oxidized by alloxan (105). As has already been pointed out the interaction between alloxan and the sulfhydryl groups of protein consists not only of oxidation, but also involves the formation of a new compound with the sulfhydryl groups of protein, and the addition of glutathione would not necessarily be expected to reactivate the sulfhydryl group (101). Furthermore, one could not expect the glutathione of the ascinar cells to be readily available to the beta cells.

### *Glutathione and Alloxan Diabetes*

Whatever the mechanism may be by which alloxan selectively kills beta cells, it is clear that the glutathione of the body will have a direct influence on the susceptibility of animals to alloxan, and the glutathione contained in the blood and the beta cells will be of critical importance. For when alloxan is administered part of it is destroyed by the glutathione of the blood and part is destroyed by other mechanisms (114, 101, 102). That which escapes destruction reaches the beta cell through the pancreatic vessels and there the glutathione of the beta cells also reacts with alloxan. If the concentration of alloxan is critical, it will not only react with glutathione but also with the sulfhydryl groups of protein and produce cell death, presumably by inactivation of essential enzymes.

It has already been shown that the injection of glutathione completely protects rats against alloxan diabetes (95, 70). Conversely it would be expected that any factor which would decrease the glutathione content of the body would increase the susceptibility to alloxan. For example it has been found that the injection of ascorbic acid, which is reported to decrease the glutathione content of the blood (118), increases the susceptibility of rats to alloxan diabetes as shown by either an increased incidence of diabetes (119) or by the development of a more severe diabetes (70). Similarly the increased alloxan sensitivity observed in starved animals (97) may well be due to the concomitant decrease in blood glutathione which occurs during starvation (120). It has also been reported that the increased susceptibility of starved rats to alloxan may be overcome by administering glucose 6 hours prior to the alloxan injection (97). In this connection it is of particular interest to note that the injection of glucose into starved rabbits is also reported to restore the blood glutathione content to normal levels in 6 hours (120). The increased alloxan susceptibility that has been observed in rats fed a low protein diet (78) may also be related to the concomitant decrease in glutathione occurring as a consequence of a low cysteine intake (121). Conversely the feeding of cysteine, which increased the sulfhydryl content of tissues, decreased the susceptibility to alloxan (122).

In the studies by Griffiths (72) on the production of hyperglycemia and glycosuria with uric acid, diabetes was produced in rabbits only when the blood glutathione level had been depleted by previously placing the animals on a cystine-methionine-deficient diet and feeding large amounts of ascorbic acid. If, however, the deficient diet was supplemented by 0.2 per cent methionine, the blood glutathione level did not fall nor did the rabbits develop hyperglycemia following the uric acid injection.

Guinea pigs have been reported to be highly resistant to alloxan (123). It has

recently been reported that the blood glutathione of guinea pigs is significantly higher (50 per cent) than that of the rabbit (124), but if guinea pigs were placed on a methionine-cystine-deficient diet they did show blood sugar responses to alloxan injection (124). Thiouracil when fed for 10 to 30 days was found to increase the free sulfhydryl content of tissue and to markedly increase the resistance of the rat to a diabetogenic and toxic dose of alloxan (122). Similarly thyroidectomy, which is also reported to increase the amount of free sulfhydryl groups in tissue, is likewise reported to increase the resistance of rats to alloxan diabetes (122).

It has been previously stated that feeding a high fat diet sensitizes rats to alloxan (78, 79). The fact that methionine but not choline will protect against the effects of a high fat diet suggests that it is a sulfhydryl factor which can modify the sensitizing effect of fat. One naturally wonders therefore whether or not glutathione can modify the diabetogenic effect of a high fat diet in the partially pancreatectomized rat or in man.

#### *Glutathione and Other Forms of Diabetes*

Evidence is rapidly accumulating which suggests that other forms of diabetes may also have a glutathione factor. The injection of various anterior pituitary preparations has been found to lower the glutathione content of tissues (125-128). And recently Conn has shown that there is a direct correlation between the production of a transitory diabetes in man, produced by injecting a purified pituitary adrenocortico-trophic hormone, and the blood glutathione level (25). In comparing the effect of two different pituitary adrenocortico-trophic preparations with different glycosuric potencies it was found that the preparation which produced the most glycosuria also produced the greatest drop in blood glutathione level (129).

Ninety-five per cent pancreatectomized rats treated with thiouracil showed a decreased incidence of diabetes as compared to untreated controls. This protection afforded by thiouracil against the development of diabetes in the partially pancreatectomized rat has been correlated with the increased tissue glutathione which was observed following the administration of the drug (122). Similarly it has been found that thiouracil has some curative effect on diabetes that has already developed, for a certain number of mild (but not severe) pancreatic diabetic rats improved on thiouracil treatment and this improvement has been correlated with the effect of thiouracil in increasing tissue glutathione (122).

A number of reports on the glutathione content of the blood and tissues of human diabetics have appeared. Although many investigators have found a decrease in blood glutathione in diabetes (130-134), some have stated that in the large majority of diabetic cases the blood glutathione is normal (135). It has also been reported that the glutathione of the tissues, and the pancreas in particular, is decreased in diabetes (133). However, in view of the fact that insulin administration is reported to increase the glutathione content of the blood and tissue (136-139), the problem of glutathione and human diabetes needs careful reinvestigation, using the more specific methods for glutathione that are now available, and taking into account the effect of insulin treatment on the diabetes.

*Possible Interrelationship Between Insulin Synthesis and Beta Cell Glutathione*

As has been stated previously, the activity of a large number of enzymes depends upon the presence of active sulfhydryl groups. The oxidation of these sulfhydryl groups (SH) to the disulfide form (S-S) results in a loss of enzyme activity. By contrast the activity of insulin does not depend upon the presence of SH groups. Although insulin contains 12 per cent cystine, all of the sulfur is in the disulfide form (S-S) (16). (In fact the addition of cysteine to an already formed insulin molecule results in a reduction of the S-S groups to the SH form and produces complete inactivation of insulin (40). Reoxidation to the S-S form partially reactivates the insulin molecule.) Since the beta cells synthesize insulin in which all of the sulfur is in the S-S form, their metabolic pathways may be adjusted to favor the oxidation of SH groups to S-S groups, and they may, therefore, have a higher oxidation-reduction potential than other cells. It has been known for a long time that the islands of

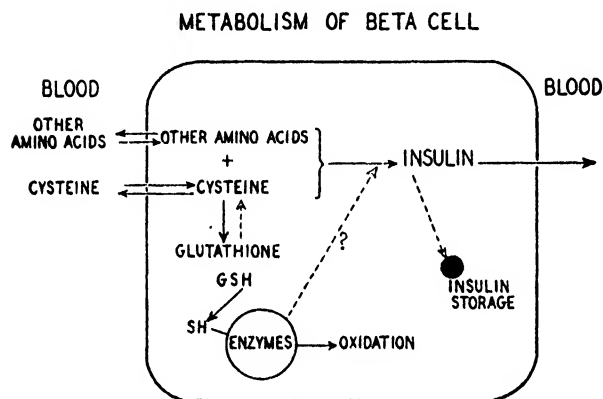


Fig. 2. SOME FACTORS INVOLVED in insulin synthesis and in the metabolism of the beta cell.

Langerhans in the pancreas can be selectively stained with Janus Green (140). When this dye is perfused through an animal, the organs, such as liver and pancreas, are initially completely stained. With time, as the dye is reduced to its leuco form, the reduction is observed to take place more rapidly in the liver and the acinar cells of the pancreas, whereas the islets remain stained with the oxidized form of the dye for a much longer time. In fact they stand out as dark blue bodies in an almost colorless or pink background. With time the dye in the islets also becomes reduced. These results, therefore, suggest that the islets do not have as great a reducing capacity as do other cells, and they may therefore have less reduced glutathione and other reduced sulfhydryl groups present than would be the case with other cells. Another consequence that would result from a high oxidation reduction potential in the beta cell would be a lessened ability to destroy by reduction certain toxic compounds such as alloxan, for when alloxan is reduced to dialuric acid it becomes non-diabetogenic (101).

It has further been postulated that the amount of glutathione present in the beta cells might also be decreased as a direct consequence of insulin synthesis (116; fig. 2). Assuming that 0.5 per cent of the weight of the pancreas is islet tissue, the

pancreas of man (average weight of 85 gm.) would contain 425 mg. of islet. Ignoring the different cell types in the islet and assuming that the islet glutathione content is equal to that of the whole pancreas (60 mg. GSH/100 gm. tissue) (105), then the total glutathione content of the islet would be 0.255 mg., and the total cysteine contained in the glutathione would be 0.1 mg. Thus if all of the cysteine contained in the glutathione of the beta cells of man could be synthesized into insulin, only 0.835 mg. or 19 units of insulin would be formed (1 mg. crystalline insulin = 22 U), which is only a fraction of the daily insulin requirement of man. Thus the synthesis of physiological amounts of insulin, by removing cysteine from the beta cell, may effectively compete with the synthesis of glutathione, or if a conversion of glutathione cysteine to insulin does take place then the synthesis of physiological amounts of insulin might reduce the glutathione content of the beta cells. It is likely that a localized beta cell depletion of glutathione may not be immediately corrected, for as previously indicated glutathione does not readily leave one cell to enter another, nor is it immediately resynthesized following acute depletion. Thus the glutathione of adjacent cells would not be immediately available to the beta cells.

A reduction in cellular glutathione would make the enzyme systems of the beta cells more susceptible to natural or administered toxic substances. Thus any factor which increases the rate of synthesis of insulin may thereby make the beta cells deficient in glutathione and more susceptible to alloxan or to other sulphydryl inactivators, and thus might influence the degeneration of the beta cells. As we have already noted 'exhaustion' and degeneration of the beta cells have been observed in the pancreatic remnant following partial pancreatectomy. Degeneration of beta cells has been observed following administration of 1) large amounts of anterior pituitary hormone, 2) thyroid hormone and 3) glucose. In all of these cases there appears to be an excessive demand for insulin. In the case of partial pancreatectomy, the pancreatic remnant is called upon to substitute for the whole pancreas (42, 43). The injection of anterior pituitary hormone markedly decreases the insulin content of the pancreas (141) as was also found to be the case in the pancreatic remnant following partial pancreatectomy (142, 143). Since the degenerative changes in the beta cells parallel the changes in insulin content of the pancreas, further support is afforded for the theory that these changes are due to overwork and consequent exhaustion of the beta cells (43, 44, 144). It is therefore suggested that these overworked cells, because of their excessive insulin production, might also show a depletion of the beta cell glutathione, and thereby become more susceptible to toxic factors and undergo degeneration.

Similarly, the feeding of a high carbohydrate diet would increase the need for insulin synthesis and in so doing might render the beta cells more susceptible to degeneration. If insulin should prove to be necessary for fat oxidation, then a high fat diet might also stimulate insulin synthesis and thereby influence the susceptibility of beta cells.

#### OCCURRENCE OF TOXIC FACTORS IN THE BODY

It may be presumed that toxic factors capable of destroying beta cells appear in the human body and thereby produce diabetes due to an insulin deficiency. How-



ever, as has been recently suggested by Conn (25), toxic factors, such as alloxan, might produce a functional impairment of insulin synthesis without necessarily producing any associated morphological change in the beta cells. What, then, are the toxic factors which may appear in the body?

### *Alloxan and Uric Acid*

The work on the production of diabetes with alloxan has caused considerable speculation as to the possible existence of a toxic factor in human diabetes (45, 114, 70). Since dialuric acid, a simple reduction product of alloxan, can be formed from uric acid by an enzyme present in dog liver (145, 146) and since dialuric acid can be readily oxidized to alloxan (114, 115) it is conceivable that abnormalities in uric acid metabolism in the human being might result in abnormal occurrences of alloxan. The production of a diabetes in rabbits following uric acid injection (72) is in harmony with the conversion of uric acid to alloxan *in vivo*. It is of interest to note that the injection of adrenocortico-trophic hormone, which produces a temporary diabetes in man, also causes an increased uric acid excretion during the time of its injection (25).

There is also a good possibility that alloxan may even be a normal intermediate in the metabolism of pyrimidines. For, on the basis of feeding experiments, a study of the urinary nitrogen excretory products, and by analogy to known *in vitro* chemical reactions, Cerecedo (147-149) concluded that uracil is metabolized through isobarbituric acid, isodialuric acid, oxalurea and finally urea. One would naturally expect that alloxan which is the next oxidation product of isodialuric would be the intermediate preceding oxalurea; Cerecedo, therefore, fed this compound but he was forced to conclude that alloxan did not behave as did these other intermediates (150). However, in light of the known instability of alloxan it is likely that very little of the alloxan fed ever entered the body as such, and that most of it was probably destroyed within the intestinal tract before absorption could take place. More recent studies on the metabolism of uracil and thymine by isotopes (151) have shown that these pyrimidines are converted to urea and ammonia in the body. However, they do not prove that the intermediate steps in the reactions postulated by Cerecedo actually do occur. The possibility exists that alloxan may be a normal intermediate in pyrimidine metabolism and this problem needs careful reinvestigation using modern isotopes techniques.

It is reported that if alloxan is present in normal human blood, its concentration is less than .02 mg/100 cc. (114). However, too much significance cannot be attached to the apparent inability to find significant amounts of alloxan in the human body. For when a dose of 40 mg/kg. of alloxan is administered intravenously into rats, no traces of it can be found in the body after a few minutes of its administration. Yet, if this rat should survive for six months in the diabetic state caused by this alloxan injection, traces of alloxan could have been found during 1/100,000 part of the animal's lifetime. In view of the extremely reactive nature of alloxan, the half life for the spontaneous decomposition of alloxan at pH 7.4 and 37°C. is slightly less than one minute (115); it would be most surprising, indeed, if appreciable amounts of alloxan were found in the human body. It is not an infrequent occurrence in bio-

chemistry that known highly reactive metabolic intermediates are extremely difficult to isolate. Thus in the case of the oxidation of the carbohydrates and fats extremely large amounts of a two-carbon intermediate, similar to acetate, are known to occur metabolically (152), yet it is extremely difficult to detect this two-carbon fragment in animal tissue (153) because of its extreme reactivity. However, if the metabolic pathway of 'acetate' is blocked, as is the case for example in fluoroacetate addition, larger amounts can be isolated (154), yet it certainly would have been fallacious on the basis of earlier work to conclude that a two-carbon fragment similar to acetate is not an intermediate in the metabolism of carbohydrate and fat.

Alloxan has also been shown to affect the beta cells of man (51). Because of the early reports that large doses of alloxan produced only slight histological changes in the beta cells of man (155) it has been stated that man is highly resistant to alloxan. However, this resistance, which was observed when the alloxan was administered by slow intravenous drip, is due to the method of administration for it has been shown that the diabetogenic dose of alloxan must be increased when the time of injection is prolonged (156). It has now been clearly established that man is as susceptible to alloxan as are other species (51). The possibility, therefore, that alloxan may appear metabolically within the human body in amounts sufficient to affect the beta cells of the pancreas is one which cannot be dismissed as readily as has been done so frequently in the past.

#### *Acetoacetic Acid*

Acetoacetic acid is an intermediate in the oxidation of fat. According to modern concepts of fat oxidation, fatty acids are broken down by beta oxidation (157a) to a number of two-carbon fragments which are then either oxidized via the tricarboxylic acid cycle or two of them condense to form acetoacetic acid (157). The oxidation of the two-carbon fragments is assumed to take place by condensation with oxaloacetate which in turn may arise from pyruvate by  $\text{CO}_2$  fixation (158). In certain washed liver preparations it can be shown that the oxidation of fat results in an almost quantitative accumulation of acetoacetic acid (159). However, if oxaloacetic acid is added, much less acetoacetic acid is formed and more of the two-carbon fragments are metabolized through the tricarboxylic cycle (160). A high fat, low carbohydrate diet might, therefore, because of the intermediates formed, be expected to favor the formation of acetoacetic acid. However, it will be recalled that Houssay and Martinez showed that not all fats were equally effective in increasing the sensitivity of rats to alloxan (78, 79). For although feeding a high lard or ox-fat diet increased the susceptibility of rats to alloxan, a high coconut oil or a corn oil diet exerted a protective effect. One possible explanation for the opposite effects of coconut oil vs. lard may lie in a slightly different metabolic pathway for these two fats. These fats differ in the amount of intermediate fatty acids which they contain. Thus coconut oil contains 19.5 per cent capric ( $\text{C}_{10}$ ) and .25 per cent caprylic ( $\text{C}_8$ ) acids (161); butter contains .32 per cent capric and .49 per cent caprylic (162); whereas lard contains practically none of the intermediate fatty acids (163). Verkade *et al.* have shown that the oxidation of the intermediate fatty acids ( $\text{C}_8$ - $\text{C}_{11}$ ) proceeds by omega as well as beta oxidation (164). Since it has been calculated that 10 per cent of the intermedi-

ate fatty acids ( $C_8$  and  $C_{10}$ ) are oxidized by omega oxidation (165), it is likely that their final oxidation products would consist of oxaloacetate as well as of two-carbon fragments. Under these conditions more of the two-carbon fragments might be metabolized by condensation with oxalacetic acid and oxidation through the tri-carboxylic acid cycle, and less acetoacetic acid would be formed, than would be the case with the higher fats where omega oxidation does not take place to any appreciable degree. This explanation might account for an increased acetoacetic acid production in animals fed a high lard diet as compared to those on a high coconut oil diet. For it has been shown that when rat liver slices are incubated with capric acid ( $C_{10}$ ) they produce less acetone bodies than is the case with  $C_4$  and  $C_6$  fatty acids (166), and that the addition of succinate or oxaloacetate also decreased the acetone body production in liver slices (167). If acetoacetic acid stimulates insulin synthesis as has been claimed (84), or if it inactivates insulin (82, 83), then a high lard diet, but not a high coconut oil diet, by increasing the demand for insulin might increase the sensitivity of beta cells to degeneration.

Another interesting possibility which bears investigation is that acetoacetic acid, or a related compound, may have direct toxic action on the enzymes in the beta cells and thereby have an alloxan-like action.

#### *Toxic Substances in Ascorbic Acid Deficiency*

The question arises as to whether or not factors may appear in scorbutic guinea pigs which are toxic to beta cells, for vitamin C-deficient animals have a diabetic glucose tolerance curve and they do show beta cell degeneration (88, 89). Scorbutic animals are reported to have an increased adrenalin content in the adrenal (88), and an impaired ability to oxidize tyrosine (168). One wonders, therefore, whether or not adrenalin or metabolic intermediates or alternates in tyrosine metabolism, are capable of affecting the enzymes in beta cells and thereby producing degeneration.

#### CONCLUSIONS

When considering the etiology of human diabetes one must bear in mind that there is more than one cause of diabetes, and that the pancreas is not primarily involved in all cases. Of particular interest is the fact that, whereas some types of diabetes may be extrapancreatic at their onset (e.g. pituitary diabetes) these may later also have a pancreatic factor because of a secondary involvement of the beta cells. Since diabetics are usually not born with their disease but rather develop it in the course of their lifetime, it is a reasonable hypothesis to assume that toxic or metabolic factors may appear in man and contribute to the progressive degeneration which is taking place in the beta cells of the human pancreas. Much work needs yet to be done to elucidate the nature and action of these toxic and metabolic factors. Many interesting leads have been reported and these await further investigation.

It should also be emphasized that the physiological state of the body, and the beta cells in particular, determines the susceptibility of the organism to diabetes. The peculiarities of the beta cells which may result from their specialization for insulin synthesis have been discussed. As was first shown in the case of alloxan diabetes,

the glutathione content of the body directly influences the susceptibility to alloxan. Evidence is rapidly accumulating which suggests that glutathione may play an important rôle in the development of other types of diabetes as well. It may yet prove to be an important factor in the development and progression of human diabetes. Much basic work remains to be done. As has already been suggested (70) human diabetes may not only be due to the appearance of abnormal toxic or metabolic factors, but it may also be due to a defect in glutathione metabolism. As more information is obtained on the nature of the factors which affect the susceptibility to diabetes, it may be hoped that we shall someday be able to prevent the onset and development of human diabetes by feeding some protective substance. Who knows but what glutathione or some precursor of glutathione may prove to be such a protective compound?

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# DIABETES AND THE INSULIN-ADMINISTRATION PROBLEM

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**I**N 1921, BANTING AND BEST aided by Collip, Scott and others, succeeded in obtaining a suitable active extract of the islet tissue, thus laying the foundation of modern diabetic therapy.

From 1921, work proceeded with the object of obtaining purer specimens of insulin and of prolonging its action. Banting and Best (10-12), Dudley (84) and Fisher (93, 94) obtained purer active extracts of the pancreas, while Abel (1) prepared the first crystalline specimen.

Although the discovery of insulin and its preparation in a form suitable for administration were of immeasurable benefit to many tens of thousands of diabetics, disadvantages were still evident. Injections had to be made frequently and this form of dosage was unpleasant; apart from this, the action of soluble insulin tended to be too dramatic. It was too quick and too short. A smoother blood-sugar curve was very desirable to minimize the risk of serious hypoglycemia.

Soon after insulin was discovered, Bliss (29), Fisher (93, 94), Macleod *et al.* (5) and Hedon (116) noted that administration of insulin was of itself insufficient to keep alive depancreatized dogs for an indefinite period of time. Sooner or later, these animals showed a marked depression and weakness, together with extreme sensitivity to insulin which led to death. On post-mortem examination, it was found that the liver was exceedingly fatty and that this was usually the only pathological condition. If raw pancreas was included in the diet, all these symptoms were prevented or cured. Hershey (118) and Hershey and Soskin (119) demonstrated that the feeding of a preparation of lecithin prepared from egg yolk produced the same effects as raw pancreas, while Best, Hershey and Huntsman (21) maintained that the choline constituent of the lecithin molecule was responsible for its physiological activity. It was observed by Ralli, Flaum and Banta (198) that the activity of raw pancreas exceeded that which could be accounted for by an estimate of its lecithin or choline content, and in 1936, Dragstedt, Prohaska and Harms (801) prepared a very active pancreatic extract which contained little choline. The active principle of this extract was considered to be a hormone and was named 'lipocaic'. Its hormonal nature was contested by Chaikoff and his co-workers (47-49), by Ralli, Flaum and Banta (198) and Ralli, Rubin and Present (199) who asserted that ligation of the pancreatic duct produced a fatty liver and that the oral administration of the external secretion of the pancreas was as effective as the feeding of raw pancreas. McHenry and Patterson (159), in reviewing the literature on lipotropic factors, concluded that it was doubtful whether lipocaic was an endocrine factor and felt that its physiological nature could be more closely compared with that of the pernicious anemia factors. Whether or not insulin as isolated and administered is the natural hormone or a precursor or derivative still remains to be solved. It is interesting to note that Wiener (249) has reported the existence of a new hormone, para insulin, related to guanidine and playing an important part in carbohydrate metabolism.

Three main lines of investigation were followed in attempts to overcome the difficulties associated with insulin therapy: *a*) attempts to prolong the action of insulin, so that injections need be given less frequently; *b*) attempts to administer insulin, or a combination of insulin with some other substance, by mouth; and *c*) attempts to obtain substitutes for insulin.

## REVIEW

*a) Attempts to Prolong Action of Insulin so that Injections Need be Given Less Frequently.* In 1923, Burgess *et al.* (40) attempted, without success, to extend the



action of insulin by the addition of 20 per cent of acacia to a solution for injection. Further attempts to obtain an insulin preparation of prolonged action were made by De Jongh and Laqueur (70), who added protein, Lange and Schoen (143), cholesterol and Leyton (154), who administered insulin in an oily medium; Skouge and Schrumph (221), who combined insulin with lecithin; Steinitz (226), insulin with desoxycholic acid, Brahn (33, 34) who added insulin to pectin solution, and Bischoff and Maxwell (28) who added to insulin such solutions as basic ferric chloride or tannic acid. Combinations of insulin with tannic acid were described also by Chiancone (50) and Lun (157); Lun stated that the presence of sodium thiosulphate enhanced the effect of the tannic acid, while Jenkinson (128) described an insulin-tannic acid-zinc suspension with a delayed and prolonged action, but which possessed the disadvantage of causing local dermatological reactions.

In 1939, Cazzani and Spiga (46), and Dogliotti and Giannini (76) described a compound of insulin, the diethylamino-sulphosalicylate, which was stated to have a prolonged and intense action upon the blood sugar and to be better tolerated by diabetics than ordinary insulin.

For the treatment of juvenile diabetes, a condition which may be difficult to control satisfactorily with soluble insulin, Feinblatt *et al.* (91) and Warburton (245) described a compound of insulin with hexamine which had a rapid yet sustained action.

Such modifications of insulin or of the solution of insulin for injection as have been described above, and many others not mentioned, proved, in general, unsatisfactory. In 1935, Hagedorn combined a solution of insulin hydrochloride with the protamine obtained from the sperm of a species of trout, *Salmo irideus*, and so obtained protamine insulin which, when injected in buffered solution at a *pH* of 7.3, gave a prolonged insulin reaction.

The duration of effect of injections of preparations of crystalline insulin was found by Altschuler and Leiser (6) and Mains and McMullen (162) to be roughly twice that of solutions of insulin hydrochloride, while the discovery by Scott (213) that insulin crystals contained zinc and that more zinc could be added to insulin during crystallization to form zinc insulin led Scott and Fisher (214) to add zinc to protamine insulin, thus obtaining zinc protamine insulin, a compound which possessed a delayed effect, coupled with a prolonged action. Thus it was maintained by Bennet (18) that zinc protamine insulin, on the non-diabetic subject, was not effective until 9 to 11 hours had elapsed from the time of injection, and that the maximum effect was not reached for 15 to 20 hours. In the diabetic, Aitken (2) stated that an earlier initial effect was produced (within 6 to 8 hours of the injection), while the total duration of effect might be as long as 60 hours, depending upon the magnitude of the dose.

The peculiar properties of zinc protamine insulin gave rise to several disadvantages in its usage. For example, in the treatment of diabetic coma, where a rapid hypoglycemic action is essential, it could not replace insulin hydrochloride due to its insoluble nature and delayed action; it tended to possess a variable rate of absorption, which might result in severe and protracted hypoglycemic reactions insidious in onset and liable to arise at night.

b) *Attempts to Administer Insulin, or a Combination of Insulin, with Some Other Substance by Mouth.* Prior to the production by Banting and Best (10-12) of an active insulin preparation, many attempts to obtain active extracts of the islet tissue of the pancreas were made. These failed, partly due to the extreme toxicity of the extracts and partly because they were often administered *per os*, a procedure now regarded as useless; but the great desirability of treating diabetes other than by injection of insulin led to many attempts being made to administer it by some alternative route.

It appeared to have been amply demonstrated that insulin was inactive when administered orally, although Rathery *et al.* (200) claimed to have obtained a positive insulin effect when they administered it in massive doses to rabbits and dogs, while Wilson (250) had consistently produced a hypoglycemic coma in mice when he administered insulin hydrochloride by mouth, in doses 90 times greater than were necessary to produce a similar effect parenterally, and had obtained the same effects with insulin digests. Wilson found, in addition, that ferric salts potentiated the hypoglycemic effects of orally administered insulin hydrochloride and insulin digests.

Most 'oral insulin' preparations were designed with the object of preventing the breakdown of the molecule by the digestive enzymes and then facilitating its absorption in the unchanged state. Samck (210) added saponins and preparations of bile, but was unable to demonstrate any hypoglycemic effect; his conclusions were confirmed later by Guiliani (110). Daggs, W. R. Murlin and J. R. Murlin (68) obtained a hypoglycemia of about half an hour's duration when they gave insulin together with hexylresorcinol and sodium carbonate. The same workers (69), after further investigation, considered, however, that the results obtained were only mildly encouraging. Lasch and Schönbrunner (146, 147) administered insulin with added saponin to aid its absorption, Trypan Red to inhibit gastric digestion and Malachite Green to prevent tryptic digestion. When insulin was administered in this way, it was noted that its activity, unit for unit, was less than when it was administered parenterally, but it was nonetheless considered useful in the treatment of diabetes mellitus. Good clinical results were obtained by Cutting and Robson (65) with insulin-quinine mixtures, but whether the beneficial results obtained were due to the unimpaired activity of the insulin or to the effect of the administration of quinine upon appetite appeared doubtful.

In general, attempts to administer insulin, in some form or other, by mouth cannot be said to have yielded encouraging results.

Sublingual administration of insulin was stated by Sachs (209) to be as satisfactory as subcutaneous; Oë (186) demonstrated an insulin effect upon the blood sugar with insulin inhalations. Insulin suppositories were administered with varying effect by Brahn and Langer (35), Wuhrmann (255) and Brahn (33, 34), and Russell, Busse and Uhl (207) applied standard preparations of insulin to the abdominal skin of rabbits, but were unable to demonstrate any hypoglycemic effect unless the skin had been pretreated by chloroform, petroleum ether or some other fat solvent; while insulin salves were used with similar results by Maier-Weinerts-Grun (161), Hermann and Kassowitz (117), Pibram (194), Mayor (166-169) and

Russell, Busse and Uhl (207). The effect of the subcutaneous implantation of pellets of insulin was investigated by Cutting (65, 66) and Parkes and Young (190). The effects of the subcutaneous administration of insulin in powder form to rabbits, via a cut in the skin, were observed by Carbonaro and Imbesi (44) and found to be similar to those obtained from subcutaneous injection.

In all of the methods of administration mentioned above, it was found that the absorption of the hormone was both incomplete and uncertain, and it appears, therefore, that they have little practical applicability, especially since precision of dosage is so very important with a remedy which has such potential dangers.

c) *Attempts to Obtain Substitutes for Insulin.* The hypothesis that wherever carbohydrates were synthesized or broken down, there must be a hormone, either identical with or having properties similar to insulin, led to many attempts being made to isolate such substances from sources other than the animal pancreas. The raw materials used were largely vegetable and were very varied; the products obtained were claimed to be active, sometimes only when administered parenterally, sometimes also by mouth. Rarely was the active principle involved identified; rarely were the claims put forward substantiated.

The knowledge that bacteria, yeasts and other microorganisms contained enzymes concerned with carbohydrate metabolism led to many attempts being made to isolate insulin-like substances from them. Shikinami (216) obtained hypoglycemic extracts from cultures of the mold *Aspergillus ozyzae*, cultivated upon moist rice. Becker (16) suggested the use of cultures of *Bacillus erodians* obtained from the intestines of dogs or doves, alone, or with the addition of a digestive enzyme in the treatment of diabetes mellitus. Administration was either *per os* or *per anum*. Little, Levine and Best (155), Winter and Smith (254) and Shikinami (216) obtained from cultures of *Bacillus coli* substances which, when injected into fasting rabbits, caused a marked reduction in the blood sugar level, but Rennesbaum (202) found that, if the lethal dose of a culture of the same bacillus was injected intravenously into rabbits, there was a marked hyperglycemia, followed in a few hours by death. Delafield (72) noted that, when a suspension of dried *Bacillus aertrycke* or the bacterial filtrate from a culture of the same organism was injected intravenously into fasting rabbits, there was a hypoglycemia of about 50 mg. per cent, preceded by a markedly hyperglycemic stage. Death occurred during the hypoglycemia. The same author (71) ascribed the preliminary hyperglycemia and the subsequent toxic effects to bacterial polysaccharides. Insulin-like effects were ascribed to bacteria by other workers. Evans (89) found that extracts of *Haemophilus pertussis*, when injected into rabbits, caused a hyperglycemia followed by a profound hypoglycemia; Hutchinson, Winter and Smith (121, 122) obtained from samples of commercial yeast a microorganism which, when cultivated, yielded an extract with insulin-like properties. Similar hypoglycemic extracts were obtained by Levine and Kolars (152) and Simola (219); Simola suggested that the active principles were guanidine derivatives, while Menten and Kipp (170) found that, when large doses of paratyphoid-B-toxin were injected into normal dogs, there was a hyperglycemia followed by a fatal hypoglycemia. In depancreatized dogs, the preliminary stage was absent. Friis-Hansen, Mortensen and Nielsen (98) obtained similar results from the injection of a lethal dose of diphtheria toxin into rabbits.

Substances said to be analogous to insulin in their action upon the blood sugar were obtained from yeast by Alzona and Orlandi (7), Barone (13), Bickel and Nigmann (24), Binet, Fabre and Bargeton (25), Brugsch and Horsters (37), Chopra and Bose (51), Collin (56, 57), Collip (58-60), Fetzer (92), Hutchinson, Winter and Smith (121, 122), Kaufmann (131-134), Maehara (160), Nagai (176), Simola (219), Shikinami (216), Von Euler (240) and Winter and Smith (251-254). The administration of yeast or of yeast extracts was also found to be beneficial in the treatment of diabetes by Beckert (17), Funk, Casimir and Schoenborn (99), Funk and Corbitt (100), Gaebler and Ciszewski (101) and Bufano (38). This group of authors did not, however, consider that it was necessary to postulate that an insulin-like substance was present in yeast, but was of the opinion that the effects of yeast, yeast extracts etc. were due mainly to their content of vitamins of the B group. Thus, Funk, Casimir and Schoenborn and Funk and Corbitt demonstrated that pigeons fed upon a vitamin-free diet became hyperglycemic, but that, when vitamin B was administered as yeast, the blood sugar fell to its normal level. This finding was confirmed by Gaebler and Ciszewski, who were able to produce a similar effect by giving the vitamins as pure substances and who did not consider that there was any necessity to assume that a 'glukokinin' was present in yeast.

Hutchinson, Winter and Smith (121, 122) considered that the activity of yeast was due not to the presence of a 'glukokinin' or to an insulinoïd substance, but to the presence of microorganisms in samples of commercial yeasts which produced the hypoglycemic substances. As previously mentioned, Hutchinson, Winter and Smith described the preparation of cultures of an organism found on commercial yeast and demonstrated that extracts of such cultures had the power of producing profound hypoglycemia, accompanied by convulsions when injected into rabbits.

Biovin (27) prepared extracts from three strains of yeasts, but was unable to obtain much hypoglycemic activity. From iso-electric point and solubility determinations, he was able to show that the extracts were not identical with insulin. Similarly, Costa (63) was not able to obtain much hypoglycemic activity from yeast, but found that, when glucose was administered simultaneously with yeast extract, the peak of the hyperglycemic curve was not reached at the normal time, but was delayed. Costa (64) later contradicted this finding by stating that hyperglycemia was caused by injection of yeast extracts.

Mushrooms were found by Glaser and Wittner (104) to contain a substance which produced a lowering of the level of the blood sugar of rabbits. This factor was identified as the enzyme tyrosinase. Binet and Marek (26) and Beauvillain (15), in investigating the toxic nature of the fungus *Amanita phalloides*, found that extracts, when administered to rabbits, caused a considerable hypoglycemia which was associated with convulsions.

It was perhaps natural that, in view of their high content of carbohydrate, some attention in the search for an insulin substitute was paid to the cereals. Collip (58-60) extracted from the roots and sprouted grain of barley substances which were claimed to produce lowering of the fasting blood sugar level of normal rabbits. This was confirmed by Donnard and Labbé (77), who used aqueous extracts of barley malt dust, and by the same authors (78) using aqueous extracts of the radicals of germinating barley from which were obtained both hyperglycemic and hypoglycemic

fractions. The use of barley and the derived malt, malt extract, infusion of malt and diastase was recommended by De Souza and Allemand (73) as an adjunct to diabetic therapy.

The use of oatmeal in the treatment of diabetes mellitus was advocated by Von Noorden (241-243), who, among others, obtained a general improvement in condition and even a cessation of glycosuria by prescribing a diet of oatmeal gruel. It was considered by Naunyn (184) that the apparent benefit was probably due to undernutrition, while Blum (30) showed that, when administered under identical conditions and in equivalent amounts, the carbohydrate of oatmeal was assimilated in the same way as was that obtained from other sources, a conclusion which was supported by Foyer (96), who found that, if anything, an increase in blood sugar level was brought about by an oatmeal diet, a not very surprising discovery!

Active extracts were prepared from oat grain by Ambis (9), Boden, Neukirch and Wankell (31), Boruttau (32), Kaufmann (131-134) and Simola (219). Ambis obtained an active preparation by an aqueous extraction of peeled, ground oat grain; Boruttau used the cortical layer of the grain and obtained a concentrate which reduced glycosuria in diabetic patients. Simola prepared an active extract from oat hulls, and considered that its action was due possibly to the presence of guanidine derivatives. Boden, Neukirch and Wankell obtained their active product from oat bran and demonstrated its efficacy by administration to rabbits, normal individuals and a diabetic patient, while Kaufmann obtained a similar effect from extracts of oats.

Best and Scott (23) prepared extracts from rice which, on subcutaneous injection into rabbits, produced a hypoglycemia. This work was not confirmed by Braun and Rees (36), who supported their conclusions by citing a private communication from Best (20).

Kaufmann (131-134) was able to prepare active extracts from rye, which, when fed to fasting rabbits, caused a reduction in the level of the blood sugar, beginning two to three hours after administration and reaching a maximum in six to eight hours; Kaufmann (131-134) also obtained active extracts from wheat and maintained that the activity of extracts of cereals increased in the order: rye, wheat and oats. Hypoglycemia-producing extracts of wheat were also prepared by Best and Scott (23) and by Collip (58-60), the latter using wheat leaf as starting material. More recently, Greiff (106) obtained from the aleurone layer of cereals, including wheat, a red pigment which acted as an oxidation catalyzer in carbohydrate metabolism and which, it was claimed, could be administered in tablet form as a substitute for insulin.

Roots and other plant organs concerned with the storage of reserve food material as carbohydrate and with its metabolism were investigated by several workers, with the object of isolating the substance which was responsible for the synthesis of storage polysaccharide and for its later degradation, when required as a source of energy. It was felt that such a substance would be similar to and might even be identical with the hormone insulin and hence that these plant organs might form a valuable source of supply of insulin or an insulin substitute.

Extracts of the green leaves and stems of the bean were made by Collip (58-

60). These were found to be active. Eisler and Portheim (87) obtained active material from dried beans (*Phaseolus vulgaris*) and Mercier and Bonnefous (171) obtained similarly active concentrates from Boubour beans (*Phaseolus radiatus*). Kaufmann (129-130) prepared from the testas of beans (*Phaseolus vulgaris*) an extract which was stated to cause a lowering of the blood sugar level of fasting rabbits. The same author (131-134) obtained an extract of bean pods which was named 'phaseolan', but which gave inconclusive results; a better extract was obtained from the actual beans and the use of this in diabetic therapy was recommended. The findings of Kaufmann, with reference to 'phaseolan', were not confirmed by Gebhardt (103), but Otto (189) obtained, from immature bean pods, a substance similar in action to insulin, while Gohr and Hillgenberg (105) obtained from the pods of the French bean an extract which reduced alimentary and adrenalin glycosuria, but which had little or no effect on the blood sugar of normal or fasting dogs.

The possibility that the common beet (*Beta vulgaris*) contained a hypoglycemic principle was confirmed by Dubin and Corbitt (81, 82), Best and Scott (23), Sammartino (211), Best, Jephcott and Scott (22) and Rychlik (208). Sammartino considered that the effect produced was due probably to vitamin content, not to the presence of an insulinoid substance or 'glucokinin'.

Dubin and Corbitt (81, 82) Ederer (85) and Macdonald and Wislicki (158) obtained active preparations from cabbage. The validity of the conclusions drawn by Dubin and Corbitt was questioned, however, by Braun and Rees (36) and by Jorgensen and Lynn (126), who were unable to confirm the results obtained, while Lewis (153) has not been able, so far, to repeat the work of Macdonald and Wislicki.

Frank *et al.* (97) obtained from a petroleum ether extract of carrots an amorphous yellow substance which was active in producing hypoglycemia when administered to man, dogs and rabbits, succeeding where Dubin and Corbitt (81, 82) apparently had failed. It may be mentioned that di Bella (74) found that intravenous injections of a solution of carotene in peanut oil produced hypoglycemia in rats, guinea pigs, dogs, hens and pigeons, but not in rabbits or cats. Best and Scott (23) and Dubin and Corbitt (81, 82) obtained hypoglycemic extracts from celery; Sammartino (211) obtained a moderately active extract from the endive (*Cichorium endiva*), but considered once again that this was probably due to its vitamin content; Glaser and Wittner (104) obtained a similarly active extract from horse radish and were of the opinion that the activity was due to the oxidase present. The Jerusalem artichoke, with its high inulin content, was considered by Shohl (217) and Carpenter and Root (45) to be a valuable adjunct to diabetic therapy, since it was thought that the diabetic organism could assimilate inulin better than other forms of polysaccharide. Carpenter and Root noted that when the Jerusalem artichoke was included in the diet of diabetics there was a reduction in the level of the blood sugar and a cessation of glycosuria, but Campbell (43) was unable to confirm this. Hypoglycemic properties were also ascribed to extracts of lentils prepared by Kaufmann (131-134). These possessed the further advantage of being active when administered orally; Collip (58-60) and Dubin and Corbitt (81, 82) obtained active concentrates from lettuce, but Jorgensen and Lynn (126) could not confirm their findings.

Extracts of the juice of the common onion (*Allium cepa*) were made by Janot and Laurin (123) and Laurin (149) and found to possess a marked hypoglycemic activity when administered parenterally to fasting rabbits. Collip (58-60) obtained active extracts from the green tops of sprouting onion plants and from onion roots and bulbs; Lalend and Havrevold (142) extracted from garlic (*Allium sativum*) and ether soluble, steam volatile alkaloidal substance which, when mixed with the disulphides found in garlic juice and injected into dogs and rabbits, gave a hypoglycemic reaction. It was not considered that this substance was analogous to insulin. Extracts of onion tops, bulbs and roots were found to be inactive by Best, Jephcott and Scott (22), Braun and Rees (36) and Jorgensen and Lynn (126).

The testa of the pea (*Pisum sativum*) yielded to Kaufmann (128-130) a very potently hyperglycemic extract, but Kaufmann (131-134) obtained extracts from whole peas which, when fed to rabbits, were effective in lowering the blood sugar level and in reducing alimentary hyperglycemia. Extracts of potatoes were found to be active by Best and Scott (23), Lalend and Havrevold (142) and Simola (219), but Hutchinson, Smith and Winter (121, 122) found that extracts prepared by them from raw potatoes were quite inactive. Raw potato juice, when administered parenterally to rabbits, was found by Thalimer and Ferry (238) to produce hypoglycemia.

Insulin substitutes have been sought in many directions other than those mentioned above. Among these, some call for special attention in view of the claims made for them.

An extract of *Scoparius dulcis*, known as 'amellin', was prepared by Nath (177) and claimed by Nath (178, 179) and Nath and Banerjee (181) to give considerable relief from the hyperglycemia, glycosuria, acidosis and acetonuria associated with diabetes mellitus. The extract was investigated clinically; administration was by mouth and was accompanied by administration of calcium salts. The properties of 'amellin' were claimed to be superior in many respects to those of insulin, although the grounds for this assertion appeared to be rather tenuous as only three patients were used in the clinical investigation. Nath (178, 179) after further investigation stated that the reduction in blood sugar level occurred in general only after initial adjustment of diabetic anemia and considered that 'amellin' was of value in improving anemias which characterized diabetes of long standing. Nath and Chowdhury (182) found that 'amellin' was superior to insulin in its effect upon the inorganic blood phosphorous level. Nath (178, 179) considered that the high cholesterol content of the blood associated with diabetes mellitus responded to treatment with 'amellin' in a very favorable manner, while Nath and Chowdhury (183) claimed that 'amellin' prevented tissue wastage and caused better utilization of protein.

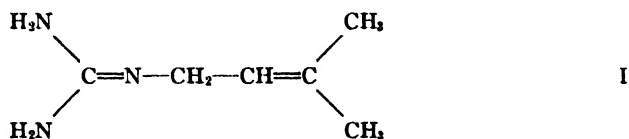
In view of the absence of confirmatory data and of the apparent retraction of the earlier claims made for 'amellin', its value in diabetic therapy appears to be suspect. Whittaker (248), in a critical survey of the work of Nath and his associates, found no evidence for supposing that 'amellin' had an important therapeutic action. Clinical evidence was cited, but was limited to reports upon two cases. Further investigation is, no doubt, necessary.

An extract of the leaves of the bilberry or blueberry (*Vaccinium myrtillis*) or

the whortleberry (*Vaccinium vitis idaea*), known as 'myrtillin', was found by Mark and Wagner (164) to reduce alimentary hyperglycemia when administered by mouth to normal dogs. Eppinger, Mark and Wagner (88) found that 'myrtillin' prolonged the life of totally depancreatized dogs and its use in the treatment of diabetes mellitus was advocated by Von Noorden-Salomon (244) and by Rathery and Levina (201). Dietering (75) was, however, able to obtain only toxic effects when extracts of *Vaccinium myrtillis* were administered to dogs and cats and found that similar effects were caused by administration of hydroquinone previously demonstrated to be present in bilberry leaves by Oettel (187). Allen (3, 4) obtained inconclusive data from trials of blueberry leaf extract, but was able to demonstrate a reduction in alimentary hyperglycemia. Allen (3, 4), Shniper (218) and Braun and Rees (36) confirmed Allen's findings. 'Myrtillin' was not, therefore, regarded as a substitute for insulin, but was thought to be a useful adjunct to it, a feeling emphasized by Watson (246), who was unable to demonstrate a marked hypoglycemia, even after massive dosage. A hypoglycemic effect upon rabbits was demonstrated, however, by Kaufmann (128) and by Edgars (86).

The use of infusions of the leaves of *Eucalyptus globulus* in the treatment of diabetes mellitus was first advocated by Faulds (90). Perez (193) obtained good results from infusions or decoctions of the leaves of other species of *Eucalyptus*. Trabut (239) confirmed these findings, but considered that benefit was due rather to the presence of tannins than to any specific antidiabetic principle. John (125) found that the infusion was quite ineffective. Preparations of *Eucalyptus* leaves are not found in present-day diabetic materia medica.

The alkaloid galegine (I), which was isolated from the seeds of *Galega officinalis* by Tanret (235) and synthesized by Spath and Spitzzy (225),



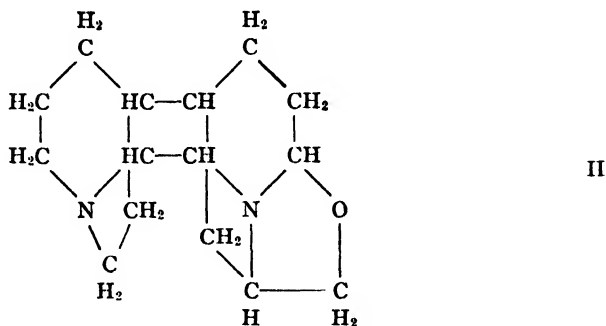
was found by Müller (175) and Simonnet and Tanret (220) to produce a lowering of blood sugar level when administered by mouth. There was, however, only a slight margin between the hypoglycemic and lethal doses and, while Parturier and Hugonot (191) considered that fluid extracts of the seeds were of value in mild and senile diabetes, the toxic nature of galegine discouraged its use.

Extracts of the seeds of *Eugenia jambolanum* (*Syzygium jambolanum*) were found by Kramer (140) and Stephenson (227) to reduce glycosuria in diabetic patients, while Mercier and Vieu-Bonnafois (172) demonstrated their hypoglycemic action. In contrast, Kaufmann (128-134) obtained only hyperglycemic extracts from the seeds and with Mercier and Vieu-Bonnafois (172) was unable to obtain active extracts from the bark. To what the beneficial effects found by Kramer and by Stephenson were due is not clear, but Jambul fruits have been used in folk medicine in India and the East Indies in the treatment of diabetes mellitus and the fresh seeds are stated to be much superior to the dried ones in this respect. According to



*Southall's Materia Medica*, the constituents of the seeds are a glycoside, antimellin, resin and gallic acid.

The alkaloid lupanine (II) obtained from the seeds of *Lupinus albus* was found by Clemanti and Torrisi (55) to possess a definite hypoglycemic action when administered either orally or parenterally to normal animals. It was found, in addition,



to reduce the hyperglycemia and glycosuria of diabetic subjects. Clemanti and Torrisi were not of the opinion that lupanine or infusions of the seeds of *Lupinus albus* were to be regarded as useful in diabetic therapy, since the hypoglycemic action was only slight and was not proportional to the dose given, even when toxic amounts were administered. Orestano (188) confirmed the slight hypoglycemic action of extracts of the seeds.

Extracts of the fresh or dried root bark of *Fatsia horrida* (*Echinopanax horridus*), known also as the Devil's Club, were reported by Large and Brocklesby (145) to reduce alimentary hyperglycemia in Belgian hares and to produce hypoglycemia when fed to fasting rabbits. Piccoli *et al.* (195) and Stuhr and Henry (228) were unable to obtain any hypoglycemic effects by administering similar preparations.

Antidiabetic extracts were sought from many sources other than those described. Among these may be mentioned: acorn shells (128-130); Agrimony (150); Alfalfa (173); *Alisma plantago* (the Water Plantain) (138); *Araba chinensis*, (141); *Atractylis ovata*, (138); *Bauhinia candians* (102); *Brachylaena elliptica* (Bitter Blaar) (19); *Bryonia cretica* (215); Burdock (*Artium Sps.*) (196); *Cephalandra indica* (Telachucha) (52); *Centaurea Salmantica* (206); *Chimaphila umbellata* (223); citrus fruits (95, 236, 237); cotton root (128-130); *Coularea lati flora* (109); *Mormordica charantia* (Cundeamor) (204-205, 197); *Geranium robertianum* (150); green stalks (92); *Gymnema sylvestra* (53); *Lampsana communis* (150); lawn grass (58-60); *Lycium chinense* (138); mulberry leaves (14, 150); olive leaves (163); *Panax ginseng* (135, 136); *Phellodendron sps.* (229, 230); *Pinus densiflora* (216); *Poa pratensis* (blue grass) (211); *Polygobum aviculare* (67); *Opuntia inermis* (Prickley Pear) (192, 107); *Rehmannia glutinosa* (138); *Rehmannia lulea* (232, 233, 231, 135, 136); *Rhizomi polygonati officinale* (174, 144); *Rhodea japonica* (114); rhubarb leaves (219); *Rosa canina* (42); *Scrophularia oldhami* (138); sesame seeds, (234); Silajit (54); *Solanum sps.* (222, 156, 39, 139); *Sophora japonica* (137); *Taxus cuspidata* (216); *Tecoma mollis* (41, 108); *Bauhinia fortificata* (Unha de Vacca) (127); *Urtica dioica* (165, 115); *Vinca sps.* (151, 185, 61, 247); walnut leaves (*Juglans regia*) (203) and White Snake Root (62).

## SUMMARY

At the outset, it must be said that, so far, there has been found no substitute for insulin. It is, of course, desirable to provide a substitute for insulin which can be administered orally, since once a diabetic patient is restored by clinical treatment to a condition approaching that of normal health, the necessity for repeated injections becomes a source of discomfort. Furthermore, self-administration of insulin requires a considerable degree of self-discipline in the strict observance of diet and time of dosage, and, perhaps more important still, demands that the patient shall sterilize his syringe, hands etc. before making the injection. It is obvious that there must exist many diabetics who are of insufficient mental caliber to perform these operations.

As has been outlined, many substitutes for insulin have been suggested and, although some of these have been used clinically, none has yielded results that are more than mildly encouraging; some have proved themselves to be most detrimental to the condition of the patient. Dosage with oral preparations in place of insulin therapy and/or diet has, in some instances, allowed the disease to progress to a more severe form and has even resulted in the onset of coma.

There does, however, appear to be no doubt that certain oral preparations have properties which alleviate some of the symptoms characteristic of diabetes mellitus. Extracts which contain tannins appear to decrease the distressing diabetic thirst; others which, due to their nauseating or narcotic properties, depress the appetite, decrease blood and urine sugar levels and reduce acidosis, but this effect is produced by the reduced intake of food. In this respect, many of the successes attributed to alleged insulinoid preparations may be attributed not to the drug but to the strict diet imposed. It appears highly probable that, in these cases, the diet alone would have an equally beneficial effect.

Although this review does not concern itself with synthetic antidiabetic compounds, some mention of these at this juncture is not, perhaps, out of place. The guanidine derivatives are the most important group of synthetic antidiabetics; among these decamethylene diguanidine ('Synthalin'), synthesized by Heyn (120), has had many claims made for its efficacy. Synthalin has a definite hypoglycemic action and, in addition, reduces the concentration of urinary ketone bodies. Its action is, however, much less powerful and is slower than that of insulin. It is potentially toxic; causing damage to the liver which may lead to serious complications such as jaundice or hepatitis. The lowering of the blood sugar level is not due to a specific hormonal effect, but to a toxic action upon hepatic tissues which deranges glycogen synthesis and breakdown.

In view of the serious consequences which may follow the clinical usage of supposedly antidiabetic substances, it is evident that results obtained from animal experiments should be adequately controlled and carefully checked. Before raising the hopes of the sufferer further, it would be well to follow rigid pharmacological investigation by adequate clinical trial.

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## NEOPLASIA IN COLD-BLOODED VERTEBRATES<sup>1</sup>

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IN THE experimental study of neoplasia, the cold-blooded vertebrates have been much neglected, perhaps because of the widespread belief that among them spontaneous tumors are rare, and consequently not readily available for investigation. But, as a matter of fact, all the main varieties of tumors that occur in man and other warm-blooded vertebrates have also been observed in cold-blooded vertebrates, and some varieties of neoplasms are actually more common in these animals than are the corresponding tumors in birds or mammals, including man.

The purpose of this review is, first, to summarize existing knowledge concerning the occurrence, the varieties, and the behavior of tumors in fishes, amphibians and reptiles; second, to give a brief account of tumors which have proved favorable material for studies upon neoplasia; and third, to discuss the lines of investigation which have been pursued. It is with the last aspect particularly that we wish to deal.

Earlier surveys of neoplasms in cold-blooded vertebrates (especially in fishes) have been given by Pick (95), Murray (90), Schroeders (113), Plehn (98, 99), Schmey (108); Takahashi (122), Thomas (127), Haddow and Blake (49), and Finkelstein (32). Lately, we have assembled, as a guide to source material, abstracts of all the reports in the literature dealing with tumors in fishes, amphibians and reptiles (111); to this paper the present review is complementary.

### SURVEY OF TUMORS IN COLD-BLOODED VERTEBRATES

*Fishes.* Tumors have been recorded in at least 114 different species of fishes, and in 15 additional species which have not been properly identified. Ninety-five of the tumor-bearing species are distributed among 45 families of teleosts, the bony fishes to which most living fishes belong. Nearly one-half of all species affected are representatives of only 5 families, namely the salmonids (*Salmonidae*—salmon, trout), the cyprinoids (*Cyprinidae*—carp, dace, minnows, chubs, barbels), the cod-

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fishes (*Gadidae*), the flatfishes (*Bothidae*), and the flounders (*Pleuronectidae*). It is not likely, however, that members of these families are especially susceptible to neoplastic processes; rather, because of their economic importance, greater numbers of these fishes are caught and inspected. The fact that tumors are also known to occur in 40 other families of teleosts, as well as in 4 families (9 species) of elasmobranchs (cartilagenous fishes such as skates, rays and sharks) indicates the wide distribution of neoplasia in different taxonomic groups of fishes. The majority of tumor-bearing individuals have been obtained from their natural habitat; a few were found in hatcheries or aquaria. Both marine and fresh water forms, of the most diverse habits, and from many different geographic areas are represented.

The varieties of tumors, and the number of different species in which they have been observed, are given in table 1. It will be noted that the neoplasms of epithelial

TABLE 1. TUMORS IN FISHES

VARIETIES OF TUMORS	NO. OF SPECIES AFFECTED	VARIETIES OF TUMORS	NO. OF SPECIES AFFECTED
<i>Tumors of Epithelial Tissues</i>		<i>Tumors of Pigment Cells</i>	
Papilloma . . . . .	16	Melanoma . . . . .	12
Adenoma . . . . .	11	Erythrophoroma . . . . .	5
Dental tumors . . . . .	4	Guanophoroma . . . . .	1
Epithelioma . . . . .	12	Xanthophoroma . . . . .	1
Adenocarcinoma . . . . .	6		
Thyroid "tumors" . . . . .	16		
<i>Tumors of Mesenchymal Tissues</i>		<i>Tumors of Nervous Tissues</i>	
Fibroma . . . . .	18	Neurinoma . . . . .	4
Myxoma . . . . .	5	Ganglioneuroma . . . . .	3
Chondroma . . . . .	8	Neuroepithelioma . . . . .	1
Osteoma . . . . .	15		
Lipoma . . . . .	7		
Leiomyoma . . . . .	4		
Rhabdomyoma . . . . .	8		
Hemangioma . . . . .	11		
Osteosarcoma . . . . .	3		
Lymphosarcoma . . . . .	8		
Sarcoma (unclassified) . . . . .	31		

and of mesenchymal origin are the predominant groups; this is equally true in mammals and birds. Tumors of pigment cells are relatively more prominent in fishes than in mammals or birds, perhaps because of the greater abundance of such cells in fishes. Few kinds of tumors of neural tissues have as yet been recorded.

In the group of epithelial tumors, all the main varieties are represented. The most common non-malignant form is the papilloma of surface epiderm; papillomas of the viscera have not as yet been reported. Adenomas are much less common; they have been found in the liver, kidney, ovary and the parabranial bodies. The latter are small organs which lie in front of the true gills; their function is unknown. Another tumor, unique to fish, occurs in the dental group, namely an 'odontoma' of the skin in a shark. The placoid scales of sharks are, phylogenetically, the most primitive 'teeth' of vertebrates. The other dental tumors correspond closely to those of mammals. The malignant epithelial tumors have been subdivided in table 1 into epitheliomas (epidermoid carcinomas), and adenocarcinomas, i.e., carcinomas

arising from glandular tissues. The former are more common, and occur chiefly on the lips, the oral mucosa, and the skin. Adenocarcinomas, like their benign variants, the adenomas, arise in the kidney, the cutaneous glands, and in the digitiform glands—structures having no homologue in the higher vertebrates. The last group of epithelial tumors given in the table, those of the thyroid, have been listed separately, for the nature of these is still undecided; most of them are undoubtedly not true neoplasms but goiters and we shall briefly discuss them in the next section.

The largest and most important group of piscine tumors are those of mesenchymal tissues. Practically all the varieties which are recognized in mammals and birds are represented. The prominence of the malignant types, sarcomas, is particularly noteworthy. In the table most of them have been left unclassified, excepting the osteosarcomas and lymphosarcomas. The preponderance of mesenchymal tumors in fishes is probably explainable on the grounds that, first, most of the tumors recorded are from regions where they would attract attention; and second, in fishes, as in certain mammals and birds, the mesenchymal tissues are more susceptible to neoplastic processes than are epithelial or other tissues. The majority of these tumors arise in the dermis, the subcutaneous tissues, the skeletal musculature and the bones; but many of the internal organs are also involved, i.e., one or more varieties of tumors are recorded in the esophagus, the stomach, the intestine, liver, kidney, testis, ovary and swim bladder.

Tumors of pigment cells occur in a considerable number of species. The most common of these tumors is the melanoma of the skin, subcutaneous tissue, or eye. Other pigment cell tumors, unique to fishes, are those which arise from the red-pigmented erythrophores (allophores), from yellow-pigmented xanthophores, and from guanophores, cells which contain guanine crystals. Although pigment cell tumors are often locally invasive and destructive, their behavior on the whole is relatively non-malignant.

Tumors of nervous tissues are represented chiefly by new growths of nerve sheaths which among certain species are very common (76). No neoplasm arising in the brain or the spinal cord has as yet been recorded, nor is there evidence of any systematic search for such tumors.

*Amphibians.* Unlike fishes, few species of amphibians are obtained in large numbers for human consumption. On the other hand, several species have for over a century been favorite laboratory animals, yet relatively few varieties of neoplasms have been reported (table 2). The inference must not be drawn, however, that amphibians are less susceptible to the development of neoplasms than are fishes; it is more likely that less intensive search has been made for them by workers who are interested in neoplasia. When some years ago a chance finding of a kidney carcinoma in a leopard frog led one of us to a systematic examination of this species, the incidence of this cancer was found to be high (69, 73).

Tumors known to occur among urodeles are carcinomas of the skin and of the testis, and fibromas and melanomas of the subcutaneous tissue. Although the number of known urodelian tumors is small, it includes two kinds which have been studied experimentally: a transmissible skin carcinoma of the newt, *Triton alpestris* (20), and a melanoma of the axolotl, *Siredon mexicanus*, which probably develops on a hereditary basis (114).

Among anura, a somewhat greater variety of tumors has been observed, all but one in members of the frog family, *Ranidae*. The epithelial tumors comprise adenomas and adenocarcinomas of the skin glands, carcinomas of the ovary, kidney, and liver. The mesenchymal neoplasms involve chiefly the extremities. One tumor, a myxoma or myxosarcoma, was observed in a large tadpole of *R. clamitans* (71).

**Reptiles.** Until recent years, tumors in reptiles were believed to be exceptionally rare, but lately several have been observed (table 3). Among turtles, papilloma of the skin is fairly common. Adenomas have been reported in the thyroid and the lung, and an adenocarcinoma in the stomach. The only mesenchymal tumor on

TABLE 2. TUMORS IN AMPHIBIANS

ORDER OF AMPHIBIA	VARIETIES OF TUMORS	NO. OF SPECIES AFFECTED
Urodeles (Tailed amphibians)	Carcinoma	3
	Fibroma	2
	Melanoma	1
Anura (Tailless amphibians)	Adenoma	3
	Hepatoma	1
	Carcinoma	4
	Fibroma	1
	Myxoma	1
	Chondroma	1
	Osteosarcoma	1
	Sarcoma (unclassified)	3

TABLE 3. TUMORS IN REPTILES

ORDER OF REPTILES	VARIETIES OF TUMORS	NO. OF SPECIES AFFECTED
Chelonia (Turtles)	Adenoma	1
	Papilloma	2
	Carcinoma	1
	Rhabdomyoma	1
Crocodilia (Crocodiles)	Papilloma	1
	Sarcoma	1
Sauria (Lizards)	Papilloma	3
	Carcinoma	2
	Chondroma	1
Serpentes (Snakes)	Adenoma	1
	Carcinoma	4
	Rhabdomyoma	1
	Melanoma	2

record is a questionable rhabdomyoma of the heart. In crocodiles, neoplasms have been observed only twice, namely, a papilloma of the skin and a sarcoma of the liver. In lizards, the tumors include papillomas of the skin, carcinomas of the skin and of the mouth, and multiple chondromas of bones. In snakes have been found: an adenoma of the stomach, carcinomas of the ovary, kidney, pancreas, and bile ducts; a rhabdomyoma of the upper labial folds, and several cases of melanoma of both the malignant and non-malignant forms. Recently, Ratcliffe (101) has studied an extremely atypical form of regenerative hyperplasia which is very common in the

pancreas of captive snakes, particularly those belonging to the *Crotalidae* (rattle snakes, water moccasins) and the *Colubridae* (pine snakes). The nature of the lesions is still uncertain; histologic appearance so frequently resembles neoplasms that Ratcliffe at the time of his report regarded them as such; but in a recent personal communication he questions his earlier interpretation. Of interest is the fact that contrary to the high incidence of this atypical proliferation in the pancreas of captive individuals, it is uncommon in snakes obtained from a state of nature. Whatever the character of the process, it merits further investigations.

*Structure and Behavior of Tumors in Cold-blooded Vertebrates; Occurrence of Metastasis.* There are no essential differences in structure between tumors of cold-blooded vertebrates and the corresponding tumors of birds or mammals. It has been claimed, however, that there are differences in biological behavior: while malignant tumors of cold-blooded animals infiltrate and destroy neighboring tissue, as do their counterparts in higher vertebrates, they are said to show less tendency to metastasize (49, 95, 99, 124, 127). Such statements are based largely upon study of neoplasms in fishes. Thus, Thomas (127) in a review of tumors in fishes, of which 40 were regarded as malignant epithelial growths, and 106 as sarcomas, found only 10 with metastatic dissemination. On the contrary, Takahashi (122) questioned the alleged rarity of metastasis in fish, and reported 3 examples among 20 malignant tumors.

It must be remembered that our information with respect to tumors in fishes and other cold-blooded animals is based very largely upon individuals caught in their natural habitat. It may well be that metastatic dissemination of a cancer so lessens the chances of survival of a tumor-bearing animal in its struggle for existence, that the process rarely comes to observation. Moreover, metastasis usually occurs in the more advanced stages of neoplastic growth; hence it may not as yet have taken place even when a large series of animals with cancer is examined. A pertinent example is our experience with the carcinoma of the kidney in the leopard frog. In our first report, based upon the study of 158 tumor-bearing individuals, no instance of metastasis was observed, and it was therefore concluded that the tumors differed from comparable human neoplasms in their failure to metastasize (69). But in a second series of 362 additional cases, metastatic dissemination was found in 22; this series differed from the previous one in that it included a considerable number of massive tumors, i.e., tumors in a more advanced stage (70). At present, over 75 examples of such metastasizing tumors have been observed by us.

It is not yet possible to generalize concerning the frequency of this process in malignant neoplasms of cold-blooded vertebrates, for not only do different kinds of cancer vary widely in their capacity for dissemination, but, with the possible exception of the frog carcinoma, we lack adequate information concerning any one kind. It may also be true that metastasis, like other biological processes, develops at a slower rate in cold-blooded animals than in man and other warm-blooded vertebrates. However, the fact that metastasis does take place has been definitely established for a considerable variety of malignant tumors in fishes, amphibians, and reptiles.

*Incidence of Neoplasms in Cold-blooded Vertebrates.* In his excellent review on the comparative study of cancer, Cramer (26) pointed out that in statements con-

cerning incidence of tumors, two important factors are often not considered; first, that the incidence varies with the age-constitution of any group; second, that the organ incidence varies greatly in different species. With regard to the first, he quotes figures for the incidence of human cancer in England, according to which only 1 out of 17,000 people below the age of 35 years dies of cancer; similarly, for mice, Bashford is said to have observed only 12 spontaneous tumors among 30,000 relatively young animals. Many other figures of like nature are given. The induction of neoplasia is an extraordinarily slow process, which in every species consumes a considerable proportion of the life span of the individual; hence neoplasms are rare in early life. While data on the incidence of tumors in relation to age are available for domesticated, laboratory, and captive wild animals, there is no such information concerning species living in a state of nature, as do most cold-blooded vertebrates. Despite this lack, the incidence of tumors in a few species has been found to be astonishingly high. Thus, the transplantable epithelioma of the lip and mouth of the catfish (*Ameiurus nebulosus*) has been estimated by commercial fishermen of experience to occur in 1 out of every 300 to 500 fish (80). All of the tumor-bearing catfish were full grown; no tumors were found in small, that is to say, young fish. As for tumors of the nerve sheaths in fish of the snapper family, *Lutianidae*, examination of many thousands of live fish and statements by fishermen indicate that as many as 0.5 to 1 per cent of gray snappers may be tumor-bearing (76). The incidence of the common mesenchymal tumors which arise in the corium of the goldfish, *Carassius auratus*, may exceed 10 per cent in certain pools, though this neoplastic disease is non-existent in the population of other pools (82). Papillomatous growth of the skin in the slippery dick, *Iridio bivittata*, is quite common; in one survey 30 examples were observed among approximately 6000 of these fish (72). The thyroid 'tumor' of salmonoid fishes, which will be discussed in the next section, may affect hundreds of individuals in a hatchery (36, 86). Among amphibians, an examination of 1800 frogs, *Rana fusca*, led to the discovery of 17 with adenomas of the skin, and 1 with myxochondroma (97). The carcinoma of the kidney in the leopard frog, *R. pipiens*, is even more common; examination of 10,317 frogs, most of them obtained from students', physiological and pharmacological laboratories, revealed that this cancer occurred in 217 per cent (73). While there was some variation in frequency in different lots, the incidence on the whole was quite constant and varied but little from year to year. In recent series, a similar high incidence has been found.

These figures indicate that several varieties of tumors are as common or more common in species of fishes or amphibians than the corresponding tumors in man.

With regard to the second factor stressed by Cramer (26), it is an established fact that tissue or organ susceptibility to neoplasia differs throughout the vertebrate kingdom. For example, in the two species of laboratory animals most often used in cancer research, mice and rats, the tissues most prone to become neoplastic are epithelial in mice, and mesenchymal in rats. In all species of mammals and birds one or several particular kinds of tumors have been found to preponderate (26, 30, 56, 100). Among cold-blooded vertebrates, tissue or organ susceptibility is quite as distinctive. In the examples given, the types of tumors named were either the only types observed, or by far the most common. Moreover, while these types out-

numbered all others in any given species, they were rare or absent in other, even closely related, species. For example, among thousands of red tai, *Pagrosomus major*, Takahashi (122) found 102 with multiple osteoma of the hemal spines, but only a single individual with a carcinoma; and while osteomas have been observed in at least 15 different species of fish, this tumor is far more frequent in the red tai than in any other species.

#### COMMON TUMORS SUITABLE FOR EXPERIMENTAL STUDIES

As shown in the preceding paragraph, a variety of tumors are common in species readily obtainable in this country. Moreover, they are adaptable to conditions which permit experimental investigations. Here, we shall limit ourselves to a brief account of the following: Melanoma of killifish hybrids, epithelioma of catfish, tumors of the nerve sheath in fish of the snapper family, a common mesenchymal tumor of goldfish, carcinoma of the kidney in leopard frogs, papilloma of the slippery dick, and 'tumors' of the thyroid in fishes. The last two are of debatable nature, and at least in many cases may represent extreme and atypical hyperplasia rather than neoplastic processes. They are included because they afford an opportunity to investigate important borderline conditions.

*Melanoma of Corium in Hybrid Killifishes.* There are few neoplasms of more debatable cell origin or of greater variation in biologic behavior than the melanomas. These neoplasms occur in all classes of vertebrates (2, 30, 56, 133); in cold-blooded vertebrates they are especially common in fishes (table 1). Willis (133) has recently stated: "Closer study of melanosis and of melanomas in animals is needed; it will surely help in the solution of some of the vexed problems of histogenesis in this field." The melanotic tumors which are prone to arise from hereditary melanosis in hybrids of the Mexican platyfish, *Platypoecilus maculatus*, and the swordtail, *Xiphophorus hellerii*, afford excellent material for the study of some of these problems. These are small, closely related fishes whose natural habitat is in various rivers of Mexico and Guatemala; they have long been popular as aquarium species. The parents in a state of nature are not known to interbreed, but hybridization readily takes place under domestication. Although tumors have never as yet been observed in the two parent species, the offspring of intergeneric mating commonly exhibit melanosis, that is to say, black patches on the surface of the body or the fins due to the presence of excessive numbers of macromelanophores in the corium and the subcutaneous tissue. The early stages of melanosis have been observed at birth in these viviparous fishes and even in embryos (42). In a considerable proportion of hybrids bred from certain races of these killifishes, localized congenital melanosis progresses to a neoplastic condition, a melanoma. Recent accounts of these tumors have been given by Gordon (40), and by Levine (65), whose papers contain references to the literature. The tumors grow slowly and usually form flattened or nodular masses, often of considerable size, beneath the epidermis of the body, the fins and the tail. Grossly, the typical melanomas are black in color, but as in other species, non-pigmented areas or nearly amelanotic tumors may develop. Histologically, the neoplasms exhibit considerable variation in their cellular pattern; some are composed predominantly of compact masses of spindle-shaped or polygonal cells, in others there is much pleomorphism.

In typical melanomas the neoplastic cells have the capacity to synthesize melanin. In the amelanotic variants this capacity has not been attained, although these tumors may be invaded by normal, non-neoplastic cells which contain melanin. There is evidence that a large pigment-forming cell, the macromelanophore, in some way incites the development of the tumors. It is probable that these cells become neoplastic and are greatly altered in structure and behavior. However, in some tumors the neoplastic elements may be derived from other cells in the corium. Most of these melanomas appear to remain non-malignant, although they may destroy neighboring tissues by pressure and through local invasion. Occurrence of metastasis has not been observed.

The melanomas of hybrid killifishes have been extensively studied with particular regard to the genetic factors involved in their development (40). The growth of the tumors in tissue culture has also been investigated (43, 44).

*Transplantable Epitheliomas of the Lip and Mouth of Catfish (Ameiurus nebulosus).* Epitheliomas of the lip and mouth have been reported in several widely different species of both fresh water and salt water fishes (111). One species of catfish is commonly afflicted with a neoplasm which bears some resemblance to epithelioma of the lip in man. At the time of our first report, 166 live, tumor-bearing catfish had been received in our laboratory during a period of two years; since then many others have been studied (80). The tumors may be solitary or multiple, and usually form large, red, fleshy masses upon the lips or the adjacent dental plates;<sup>3</sup> some become so massive as to prevent closure of the mouth. In approximately one half of the cases, both upper and lower lips or dental plates are involved; usually such tumors are in direct apposition. Histologically, the tumors consist of closely packed masses of epithelial cells, often in papillary arrangement, and supported by a delicate but richly vascular connective tissue stroma. In their early stages, most of them grow in an outward direction and show little sign of invasion. Later broad pegs of tumor cells may push deeply into the subjacent tissue. The larger growths are still more frankly invasive; the deeper layers of cells lose their compact arrangement, and frequently extend into blood vessels where they form emboli. The course of the tumors varies, and in most, growth is slow but progressive, while in some, increase in mass is rapid. Despite the frequency with which tumor emboli are encountered, no metastases have as yet been found.

There is but little information at present concerning the geographic distribution of this neoplastic disease other than that it is common in streams and ponds of Pennsylvania and New Jersey.

The ease with which catfish can be adapted to laboratory conditions makes possible prolonged study of the naturally occurring neoplasms, particularly with respect to their inception and course. In the catfish neither the normal nor the neoplastic mucosa undergoes keratinization; hence in their earlier stages these epitheliomas are relatively translucent; this allows study of the relation of blood vessels to the inception and progress of the cancer. It is also possible to transplant the neoplasm into the anterior chamber and between the layers of the cornea.

<sup>3</sup> The dental plates of catfish lie in immediate contact with the lips. They are flat ridges, slightly elevated above the remainder of the oral mucosa which covers them. The ridges support multiple rows of tiny primitive teeth which barely project through the mucosal surface.

*Tumors of Nerve Sheaths in Fish of the Snapper Family (Lutianidae).* These tumors closely resemble a complex group of neoplasms which hitherto have been studied chiefly in man. Reflecting opinions concerning their histogenesis, such tumors are variously called: neurinoma, neurilemmoma, schwannoma, and perineural fibroma. They are common in several members of the snapper family; especially the grey snapper, *Lutianus griseus*, the dog snapper, *L. jocu*, and the school master, *L. apodus* (76). These are large fishes, averaging several pounds in weight, which are widely distributed in subtropical and tropical waters, and are abundant around Florida and the West Indies. The tumors generally occur along the course of the larger subcutaneous nerves, and project outward as firm masses, ranging in size from small nodules to growths over 5 cm. in diameter. As a rule only a single tumor is present, but two or rarely more may occur. Histologically, they are usually composed of two kinds of tissue, one compact and richly fibrocellular; the other loose, reticulated and less cellular. The constituent cells and intercellular fibers of these tumors appear to be essentially the same, and are arranged in similar patterns in fish and in man. Palisade formations of nuclei and fibrils, which when present are regarded as a highly characteristic feature of nerve sheath tumors in man, occur in approximately one-third of the fish tumors. The growths, though well circumscribed, are usually not encapsulated, and a small number of them show limited invasiveness.

Nerve sheath tumors appear to be considerably more common in fish of the snapper family than in man. Their occurrence in a species which can be maintained for long periods in marine aquaria, renders these tumors favorable material for the study of an important group of neoplasms.

*Common Mesenchymal Tumor of the Corium of Goldfish (Carassius auratus).* These tumors belong to the group which has been named, rather loosely, fibrosarcomas; their histogenesis is still uncertain. They appear to have a wide geographic distribution, having been reported from countries in America, Europe and Asia (81). The tumors arise in the corium or the subcutaneous tissues and project outward as solitary, less often multiple, ivory-white, firm masses. While their rate of growth is generally slow, some rapidly attain relatively great size. The smaller tumors are circumscribed, though not encapsulated, whereas the larger are frankly invasive and locally destructive. Histologically, they resemble neoplasms of connective tissue. The small tumors are similar to fibromas, the larger rapidly growing tumors look like fibrosarcomas, but the paucity of fiber formation makes a purely fibroblastic origin doubtful. It is characteristic of this neoplastic disease that it occurs in certain pools and is absent or rare in others. Where prevalent, the number of tumor-bearing fishes as a rule gradually increases. The ease with which goldfish can be maintained in the laboratory, and the exposed position of the tumors, permit study of the natural history of the neoplasms, particularly with respect to their inception and course.

*Carcinoma of the Kidney of the Leopard Frog (Rana pipiens).* This is probably the most readily available tumor known at present in cold-blooded vertebrates (69, 70, 73). Over 1200 have been studied in this laboratory.

The tumors occur in one or both kidneys as solitary or multiple, white, solid or partially cystic growths varying in size from small nodules to large, irregular masses several times the size of the kidney which they replace. The larger and presumably more rapidly growing tumors not uncommonly metastasize, especially to the liver



and lungs. Histologically, the majority of the tumors have the appearance of adenocarcinomas. The component epithelial cells are atypical, much larger, and more basophilic than normal kidney cells. Usually they are crowded in disorderly multiple layers around irregularly shaped gland-like acini. Numerous mitotic figures denote active proliferation. The stroma is scanty and poorly vascular. A capsule is lacking, and marginal extensions of the tumor infiltrate and destroy the adjacent kidney.

In a smaller group the component cells are less atypical, the tumor tubules are single-layered, more orderly, and there are few mitotic figures. While a capsule is lacking, no actual invasion occurs at the periphery. A frequent variation from this adenomatous growth is cystic dilatation with papillary projections into the cyst.

All gradations are found between the frankly malignant, invasive and destructive adenocarcinoma and the structurally benign adenoma, cystadenoma and papillary cystadenoma. In general the larger tumors nearly always have a malignant appearance, although minute nodules are also often carcinomatous.

One outstanding characteristic of the frog tumor is the frequent presence of acidophilic intranuclear inclusion bodies which, in their general appearance, are like those found in herpes and certain other diseases known to be due to viruses. They are invariably confined to the cells of the tumor and have never been observed in normal renal epithelium of tumor-bearing kidneys, nor in the cells of other organs.

The cancer of the frog appears to be more prevalent in the northern New England states and in the adjacent parts of Canada than in other regions, but tumor-bearing frogs have also been obtained from Indiana, North Dakota and the Mississippi Valley. This tumor has proved suitable for various experimental studies which are discussed in the next section.

*'Papilloma' of the Skin in the Slippery Dick (Iridio bivittata).* There is no sharp dividing line between 'true' neoplasms and certain exaggerated forms of hyperplasia. Indeed the latter may merge by ill-defined stages with the former. A tumorous condition which belongs in this border-line group commonly affects a little reef fish, which is abundant in the waters around Florida and the West Indies (72). The growths generally appear as localized, flattened, nodular elevations of the skin, and frequently attain relatively large size. Histologically, they are composed of masses of atypical epidermal cells, arranged in alveolar grouping, and supported by a scanty stroma, giving a papillomatous character to the growths. The corium is infiltrated but no extension into deeper parts have been observed. Destruction of one or several fins often takes place.

Fish affected with these papillomatous growths can readily be kept in indoor marine aquaria. They should prove suitable for the investigations of a variety of problems concerning abnormal proliferation, such as the effect of carcinogenic agents on a tissue already in a state of atypical proliferation.

*Thyroid 'Tumors' in Fishes.* This is a common condition which has been variously called 'throat tumor', 'gill disease', carcinoma of the thyroid, and endemic goiter or endemic thyroid hyperplasia. It affects especially members of the salmoid family, such as various species of trout and salmon, but it has also been found in a few kinds of marine fishes. The disorder is nearly always confined to captive fish in hatcheries or in aquaria. It seems to be distributed throughout the world, though

its incidence varies from sporadic cases to many hundreds in a single hatchery. The condition is characterized by atypical and marked proliferation of the thyroid leading to the development of conspicuous tumors which protrude through the floor of the mouth, in the gill regions, and elsewhere. Histologically, the structure of the growth is complex and ranges from simple hyperplasia to the formation of circumscribed nodules composed of less well differentiated cells. Such tumors are similar to those commonly observed in mammalian goiters, and are generally regarded as adenomas. In a more advanced stage, a definitely malignant appearance may be attained, the growth being composed of solid masses of atypical cells which seemingly invade and destroy the neighboring structures, such as bone, cartilage, muscle and walls of vessels. Often no sharp distinction, on histologic grounds, can be made between a purely hyperplastic condition, a non-malignant tumor, and an invasive cancer. Classification of these growths is complicated by the fact that in most fishes the thyroid is not an encapsulated organ as in the higher vertebrates, but consists of widely scattered tissue. Two principal views are held regarding the nature of the thyroid disease: one that "The so-called carcinoma of the thyroid of brook trout is, in its early stages at least, nothing more than severe endemic goiter. The possibility that this physiological hyperplasia may, in some instances, go over into actual carcinoma is not denied and is in harmony with the more modern view of cancer development." This represents the conclusions of Marine (86) and perhaps the majority of investigators. On the contrary, Gaylord (36) and a number of others hold that, while in many of the phases, the disease is identical with endemic goiter, it frequently tends to become a cancer.

Because of the economic importance and the theoretical interest of this condition, it has been widely studied, particularly as to its etiology. Again two opposing hypotheses have emerged, one favoring the infectious nature of the process, the other regarding it as a reaction to abnormal food and over-crowding of fishes. For details, reference should be made to the monograph by Gaylord and Marsh (36), the papers by Marine and his associates (85, 86), and the review by Renaud (103).

It is of interest to note that susceptibility to this condition varies considerably among different species of salmoids, some being entirely resistant. Among marine fishes, the shark-sucker, *Echineis naucrates*, appears to be a highly susceptible species. We have been able to study the development of large thyroid growths in 16 individuals of this species kept in captivity in a large public aquarium. None of the other marine fishes maintained in the same aquarium have developed the condition during a period of over 20 years (111).

The thyroid 'tumors' in fishes probably correspond to similar growths in several species of mammals; see Willis (133) for references. They all raise the question of the relation between long persistent hyperplasia, which in some stages is reversible, and the development of non-reversible neoplasia. It would seem promising to re-study these tumors of fishes in view of the advances that have been made in the field of endocrinology.

#### EXPERIMENTAL STUDIES UPON NEOPLASIA IN COLD-BLOODED VERTEBRATES

In addition to the theoretical interest which tumors in cold-blooded vertebrates have, we may now consider them as material for the experimental study of neoplasia.

Although in number and variety such studies are as yet restricted, for many kinds of investigations cold-blooded animals have decided advantages. As compared with warm-blooded animals, their various vital processes usually are carried on more slowly; they are more closely dependent upon such environmental factors as temperature; their cells and tissues have a longer survival period *in vitro*; in some groups, particularly in amphibians, the cells, whether normal or neoplastic, are much larger than the corresponding cells of higher vertebrates. These various attributes permit a greater range in certain experimental conditions than is possible with birds or mammals.

The pertinent investigations will be taken up under the following headings: etiology, transplantation, explantation, morphogenesis, effect of temperature, induction of neoplasia, and biochemical studies.

### *Etiology*

1. *Genetic Factors.* a. *Melanoma in hybrid killifishes.* The study of hereditary factors influencing the development of neoplasms in fishes was introduced by Haüssler (52) in 1928 who observed the occurrence of melanosis and of melanotic tumors in platyfish—sword-tail hybrids. His work was soon confirmed by Kosswig (62, 63) and has been greatly extended by Gordon and his associates (40). Gordon's studies are of particular importance since they have been conducted mainly with pure strains of these fishes. He has demonstrated by genetic methods that a certain kind of pigment cell, the macromelanophore, is responsible for the production of the tumors. Normally, the diverse color patterns of most races of the platyfish are mainly produced by various arrangements of two types of melanophores, the macromelanophores and the micromelanophores.<sup>4</sup> Gordon has shown that the development and distribution of the larger pigment cells is controlled by five genes, probably multiple alleles. Each gene is dominant, sex-linked, and does not by itself produce tumors in pure lines of platyfish. When, however, platyfishes of certain genetic constitution are hybridized with swordtails, which have a fairly uniform color pattern and carry only micromelanophores, fifty per cent or more of the offspring develop melanosis. As has been discussed in the preceding section such melanosis—a congenital malformation—may progress to the formation of melanotic tumors. The development of the neoplastic state is dependent upon the interaction of the platyfish macromelanophore genes with at least two modifying genes derived from the swordtail; these latter genes are not alleles. The effect of the modifiers can be enhanced or diminished by suitable back-crossing of hybrids to parent species. As has further been shown, the amount of pigment elaborated by the neoplastic cells is also genetically controlled, and is a reversible process. It seems to depend largely upon genetic

<sup>4</sup> In a recent review Rawles (102) discusses the origin of melanin-forming cells, which she designates melanophores. They are specialized, branched, wandering cells which morphologically are similar in all vertebrates. Their less differentiated precursors are termed melanoblasts. It is in this sense that Gordon and his associates have used the term macromelanophore and micromelanophore for the melanin forming cells of killifishes. Melanin which has been liberated either by extrusion from melanophores or by death of these cells may be engulfed by any cell which has phagocytic capacity; such a cell may be termed a melanophage. There is, unfortunately, no uniform usage with regard to melanin-containing cells, and opinions differ as to their origin (88).

factors whether the neoplastic transformation of actual or potential pigment cells results in the formation of typical black melanomas, or of more or less colorless amelanotic tumors. That the macromelanophores and not the micromelanophores are concerned in the initiation of the neoplastic process was demonstrated as early as 1931 by Gordon. He interbred varieties of platyfish which do not carry the larger pigment cells (as most varieties do) with swordtails, and the offspring, although abundantly supplied with micromelanophores, failed to develop either melanosis or melanotic tumors. On the contrary, if a variety of platyfish having only macromelanophores is mated with a 'golden' swordtail which is wholly lacking in melanin-pigment cells, half the hybrids develop melanosis, i.e., the initial stage of melanomas.

*b. Ocular tumors with exophthalmia in xiphophorin fishes.* In 1939 Jahnelt (57) reported the occurrence of ocular tumors in a number of swordtail siblings, *Xiphophorus hellerii*. The growths were markedly invasive, infiltrated the muscles and even penetrated the skull. Histologically, they were composed mainly of small round cells with an admixture of spindle-shaped cells, none were pigmented, and mitotic figures were common. The author believed that the tumors arose from the choroid. He regarded them as sarcomas (perhaps they actually represented amelanotic melanomas), and suggested a hereditary basis for their origin.

Some years later, Levine and Gordon (66), in xiphophorin fishes of various breeds, which were totally unrelated to Jahnelt's stock independently discovered ocular tumors which also originated in the choroid. The growths had remarkable similarity with those reported by Jahnelt with whose work Levine and Gordon were then unfamiliar. As many of the cells were melanin producers the tumors were considered to be melanomas, comparable to those of the human eye. More recently Gordon (41) studied the genetics of these tumors, and came to the conclusion that their development appears to be controlled by interaction of two complementary autosomal genes both of which are recessive.

*c. Erythrophoromas in xiphophorin fishes.* Tumors arising from erythrophores (allophores), cells which form red pigments, have been reported in 5 species of fishes. These neoplasms have no counterpart in man or other warm-blooded vertebrates. The most detailed account of them has been given by Smith (119). In 1929 Kosswig (62) reported such tumors in two hybrid platyfish. In subsequent experiments he obtained a similar tumor in swordtail hybrids (63). He also found a typical erythrophoroma in a platyfish-swordtail hybrid, and when this hybrid was crossed with a pure line *X. hellerii* tumors were obtained in five of the offspring. Kosswig concluded that erythrophoromas developed on a hereditary basis.

*d. Melanoma of axolotls.* This is the only known amphibian tumor which probably arises on a hereditary basis. In 1932, Sheremetieva and Brunst (114) found among hundreds of axolotls, *Siredon mexicanum*, a male and a female which had minute areas of melanosis which gradually progressed into melanotic tumors. The tumor-bearing pair was mated, and several offspring developed, at first, melanosis, and later melanomas. In three successive generations during the six following years, a considerable number of the offspring again developed melanomas. In the majority of individuals, the neoplastic disease manifested itself at about the end of the first year of life as one or several pigmented areas. Some of the melanotic

areas remained unchanged, while others thickened and gradually became transformed to large tumors, several centimeters in diameter.

The melanomas vary in invasiveness, some remaining sharply circumscribed, while others extend deeply into the subjacent musculature. Histologically, their composition is very similar to that of the melanomas of hybrid killifishes. As in these fishes, the melanomas of axolotls begin in the corium, and the cell of origin appears to be a melanophore. This tumor is of interest not only because of the probable operation of genetic factors in its development, but because it is transplantable.

The breeding experiments of the Brunsts were unfortunately interrupted by the war, and the original colony of the tumor-bearing axolotls was lost.

2. *Viruses*. As long ago as 1908, the presence of inclusion bodies suggested the possibility of a virus etiology in two common papillomas of fish. Keysselitz (59) studied papillomatous tumors of the lips of barbels, *Barbus fluviatus*, of the Mosel River. The nuclei of many of the neoplastic epithelial cells contained inclusions, which the author compared to the inclusions of known virus diseases. A few years later, Breslauer (12) observed similar papillomas in another species, the stint, *Osmerus eperlanus*, from an inlet of the Baltic sea. In these tumors, cytoplasmic inclusions were sometimes, but not regularly, encountered. So far as we are able to learn, neither of these studies was carried further. Because of the fact that epidermal papillomas of at least two species of mammals (rabbit, dog) are known to be caused by viruses, the papillomas, which are the most common of the epithelial tumors of fishes, deserve further investigation.

a. *Transmissible epithelioma in the newt*. Carcinoma of the skin, believed to be caused by a virus, was reported by the Champys in 1935 (20). They had maintained colonies of newts, *Triton alpestris*, for years without having observed any new growths. In 1931, however, 3 out of 5 newts in one aquarium simultaneously developed cutaneous tumors in the tail and flank. These were found at biopsy to be typical carcinomas, invading the muscles almost down to the vertebral column. The possibility that a carcinogenic agent might be present was investigated by placing in this aquarium after removal of the tumor-bearing animals but without change of water 8 other tritons (4 *T. alpestris*, 2 *T. cristatus*, and 2 *T. palmatus*). The animals were left for one month when they were replaced by others, and so on. Of the first lot, 3 of 4 *T. alpestris* developed tumors after 18 months, but none arose in other species, nor in newts placed in the aquarium after the first month.

The tumor was propagable by inoculation, but only under special conditions. When grafts were implanted subcutaneously, they usually regressed, but approximately two years afterwards cutaneous tumors appeared in several animals, usually at some distance from the site of implantation. New growths developed only in *T. alpestris*, not in any of the other species inoculated, not even in *T. cristatus* which can be crossed with *T. alpestris*.

The experiments were interpreted as indicating the existence of a virus, which is species specific, and which may be spontaneously transmissible. No further studies on this subject appear to have been published.

b. *Frog carcinoma*. As stated above, one of the outstanding characteristics

of the kidney carcinoma of the leopard frog is the frequent presence of acidophilic intranuclear inclusions such as suggest the presence of a virus (69). Transmission experiments made it very probable that this carcinoma is, in fact, induced by a virus (73). A total of 810 frogs, in groups of 10 to 40, received inoculations from 44 different tumors. When inoculated as cell suspensions or as living fragments into the lymph sacs, the cranial cavity or the coelomic cavity, no significant local growth usually resulted, and the implanted material became resorbed. However, in over 20 per cent of the frogs surviving inoculation for more than 6 months, tumors identical with spontaneous neoplasms developed in the kidney.

Similar results were obtained after intraabdominal injections of desiccated or glycerinated tumors. As in the preceding series, kidney tumors occurred in approximately 22 per cent of the frogs that survived for more than 6 months. From these experiments the conclusion is warranted that the tumor-inducing agent can resist conditions incompatible with viability of animal cells; and that the causal agent of the frog carcinoma is very probably an inclusion-forming, organ-specific virus. No attempts have as yet been made to repeat these experiments with filtered tumor material.

As controls, 953 frogs were maintained under precisely the same conditions as prevailed in the experimental series. In these groups, there was noted a slight rise in incidence of renal tumors, from 2 per cent to 6 per cent in frogs kept for more than 6 months. While this rise is far below the striking increase in the experimental groups, it may have considerable significance. The possibility exists that the neoplastic disease is transmissible from frog to frog. In captivity, frogs are of necessity maintained under somewhat crowded conditions, thereby favoring not only direct contact but indirect transfer of various agents. Experiments were therefore devised to test the possibility that the tumor-inducing agents may be transferred by means other than inoculation. Frogs with large palpable kidney tumors were placed among groups of presumably healthy individuals; when a tumor-bearing frog died, it was replaced by another. Thus for approximately two years, normal frogs were kept together with tumor-bearing individuals. The results were inconclusive. It should be remembered that transfer of a virus disease from animal to animal sometimes requires a complex cycle (117). In the case of the frog tumor these conditions are as yet unknown.

3. *Environmental Factors of Unknown Nature.* In fish kept in captivity, some kinds of tumors tend to be prevalent among the inhabitants of certain pools or aquaria, whereas they are rare or absent in the population of others. For example, Fiebiger (31) observed the development of multiple cutaneous papillomas among a group of climbing perch, *Anabas scandens*, which had been kept in the same aquarium for two years. He likened the growths to infectious warts of mammals. Recently, Nigrelli (92) reported that all of 12 full-grown pike, *Esox lucius*, kept in one tank at the New York Aquarium succumbed during a period of two years from lymphosarcomas of the kidneys. Similarly, the common mesenchymal tumor of the goldfish, *Carassius auratus*, not infrequently affects a number of individuals in a pool (89, 106). Thus of 30 goldfish bearing such tumors, studied by us, 28 were obtained from 3 small concrete pools (81). Since hereditary factors could be ruled out, it seemed

probable that these pools provided an environment peculiarly suitable for induction of the neoplastic process. But experiments so far have indicated that if tumor-inciting factors are present they are either weak or very slow in their action. Nevertheless, the not uncommon development of tumors among fishes living in the same environment provides opportunities for investigating this problem of carcinogenesis.

### *Transplantation*

1. *Tumors of Fishes.* Until recent years the few attempts to transplant tumors of fishes were uniformly unsuccessful. The causes of the failures are obvious when the kind of tumor employed, and the techniques used, are examined. In most cases the neoplasms were benign and hence difficult to transplant even under the most favorable conditions; in all cases the number of recipient animals was wholly inadequate. Moreover, many of the recipients died long before the transplants could possibly have become established. Thus, without success, Takahashi (122) attempted to transplant a papilloma, a fibroma, and a spindle-cell sarcoma; Picchi (94), a neurinoma; Eguchi and Oota (29), a fibroma; Beatti (5), a sarcoma; Freudenthal (34), a fibroma; Haddow and Blake (49), a hepatoma; Montpellier and Dieuzeide (89), a fibrosarcoma; and Lucké (76), a neurinoma. None of these unsuccessful experiments permits general conclusions as to the transplantability of piscine tumors. Successful transplantation has recently been accomplished in two species.

a. *Transplantable epithelioma of catfish.* These tumors are readily transplantable into the anterior chamber of the eye, and, by taking advantage of an anatomic peculiarity, between the layers of the cornea (80). In these locations, grafts from most of the tumors soon become established, and the growth of the transplants can be followed by direct microscopic examination. In the first group of experiments, autotransplants of 14 epitheliomas were made into the anterior chamber and of these, 10 showed progressive growth. Usually, attachment of the graft occurred within a week and by the end of the second week vascularization had begun. The pattern of growth was relatively simple; the proliferating cells from the implants spread over the under surface of the cornea as dense membranes which gradually thickened, until by the end of the second month the chamber was filled with a compact, well-vascularized mass of tumor. Occasionally, the iris was invaded and in several cases the growing tumor extended through the operative scar and protruded beyond the cornea. In one case the growth pushed into the posterior chamber.

In another group of experiments, tumor fragments were introduced between the layers of the cornea. In the catfish, and certain other cold-blooded vertebrates, the cornea can readily be separated into two layers, of which the inner is continuous with the sclera, and the outer with the skin. By a simple operative procedure a small pocket can be made within the cornea; implanting tumor fragments into this pocket has the advantage that they are fastened between layers of a transparent tissue. Of 7 autotransplants made in this manner, 6 grew progressively. Similarly when homotransplants were made into 42 non-tumor-bearing recipients 64 per cent grew well. The corneal transplants became vascularized promptly and grew expansively, at approximately the same rate as those in the anterior chambers. Within less than 2 months a compact mass of tumor greatly distended the cornea, pushing

its outer layer forward. The growing mass achieved room by further splitting of the corneal layers. With maximal distention of the cornea, growth became slow or ceased entirely.

Repeated attempts to transplant the catfish epithelioma into the anterior chamber of alien species of fish, or of frogs, were unsuccessful. Differences, however, were noted in the local reaction of the recipients to the foreign tumor, and in the fate of the latter. In alien species of fish (goldfish, sunfish) implantations of the catfish tumor led to no exudative reaction, and the aqueous humor remained clear. The implants apparently survived for as long as 4 weeks. On the contrary, when transplants were made to members of another class, leopard frogs, an abundant leukocyte exudate promptly formed, and the transplants were soon devitalized and were resorbed entirely within 3 weeks.

*b. Mesenchymal tumors in goldfish.* Less successful than the above were experiments with goldfish tumors; they nevertheless brought out points of interest (81). Fragments of 6 of these tumors were implanted into the subcutaneous tissue or into the anterior chamber of the eyes of 99 unselected goldfish. In no case was any growth noted in a homotransplant.

Autotransplantation to the anterior chambers of both eyes was carried out in 4 fish. In one of these, vigorous and prolonged growth was attained. The rate of growth of the transplants closely paralleled the rate of growth of the primary tumor, thus suggesting the existence of host factors which operate on both the primary tumor and the autotransplants.

An analysis of the experiments with goldfish tumors brings out a relationship, long known from similar experiments on mammalian tumors. Five of the 6 goldfish tumors used were growing rather slowly; they showed only slight invasive properties and had not metastasized. Such tumors are not easily transplantable. The other tumor, however, was more invasive, and grew rapidly, and its autotransplants likewise grew progressively.

*2. Tumors of Amphibians.* Two kinds of transplantable neoplasms are at present known, a melanoma of the axolotl, and a carcinoma of the leopard frog. In addition, Gheorghiu (37) has carried a 'tumor' of *Rana esculenta* through 12 generations. About 80 per cent of the grafts became established and grew rapidly for from 5 to 20 days, then ulcerated, and the frogs died probably from infection. The nature of this tumor is uncertain. Gheorghiu regarded it as a sarcoma, but Cramer (25) believed it to be an infectious granuloma. Another, but unquestionably true neoplasm, is the epithelioma of the newt, *Triton alpestris*, which was studied by the Champys (20); we have already discussed this tumor in connection with its possible viral origin. Among amphibians, tumors which did not prove transplantable are: adenomas of the skin of an unnamed species of frog reported in 1868 by Eberth (28) who first recorded tumors in amphibians; multiple adenomas of the skin of another unnamed species of frog, reported by Pentimalli (93); adenocarcinoma of the skin of *R. esculenta*, reported by Masson and Schwartz (87) and a 'hypernephroma' of the kidney in the same species, reported by Carl (19). From the descriptions, it is questionable whether any of these tumors were malignant.

*a. Melanotic tumor in the axolotl.* The axolotl has long been a favorite animal



for studies on regenerative processes. The discovery of a transplantable tumor in the axolotl by the Brunsts (114) makes it possible to investigate simultaneously both regenerative and neoplastic processes. Reference has already been made to the hereditary basis of the neoplasm and its structural characteristics. The technique of transplantation is not difficult; small portions of the tumor, together with covering epiderm are implanted onto an area denuded of skin, or the grafts may be implanted into regenerating parts. The transplants are anchored into place by silk thread, and most of them become established. The recipients need not be genetically related, and either gray or albino axolotls may be used. Since the tumors are black they contrast sharply with surrounding tissue. Hence, it is possible to observe even the early stages of outgrowth, when pigmented neoplastic cells creep into the neighboring tissue gradually displacing normal cells. The rate of growth of the transplanted tumors is usually greater than that of the spontaneous tumors. The grafts may attain relatively large size and they in turn may be retransplanted.

A similar or identical tumor of the axolotl was studied in 1916 by Krontovsky (64) whose attempts to transplant the neoplasm were unsuccessful.

*b. Frog carcinoma.* The carcinoma of the kidney of the leopard frog is easily established in the anterior chamber of the eye. The suitability of this site for tumor transplantation was suggested by van Dooremaal (130) as early as 1873. (See Lucké and Schlumberger (77), for a historical account.) One of the advantages of the method is that the rate and manner of growth can be observed directly through the thin transparent cornea and such direct observations can be supplemented by photographs taken periodically, which serve as objective and permanent records. There is at present no other method which permits such continuous observation of a living tumor, and which gives as vivid and three-dimensional a picture of the growing mass. Another advantage is that the ocular fluids have a high degree of tolerance for heterologous tumor grafts. Systematic studies using this technique have been made by Greene (45) on various tumors of warm-blooded animals, and by Lucké and Schlumberger (77, 78, 80) on tumors of cold-blooded vertebrates.

The technique of implanting tumor fragments is simple. Using aseptic precautions, an incision 2 to 3 mm. in length is made along the upper border of the sclero-corneal junction, care being taken not to cut into the iris and thus bring about bleeding. Through the incision small bits of tumor, 1 to 2 mm. in diameter, are introduced by means of a finely pointed forceps; the transplant is then moved to any desired part of the chamber by gently stroking the surface of the cornea with a rod.

On the average, over 50 per cent of the initial transplants of a renal carcinoma of the frog grow readily. Occasionally such a tumor is found to be non-transplantable. Within a week, usually, the transplants become attached to the iris, lens, or more rarely to the cornea. The pale color of the tumor contrasts sharply with the dark background furnished by the iris. Even before growth becomes evident, there is a distinct difference between those transplants which will persist and those which will regress, the former remain translucent, the latter become opaque. In general, growth follows a similar pattern, which is independent of tumor strain or of the external temperature. A variable lag period, usually lasting 5 to 10 days, is followed by a period of gradual outgrowth from the implant. Growth thereupon proceeds

at a rapid rate until most of the anterior chamber is filled, after which the increments become smaller and smaller. When the transplanted tumor has attained considerable size, it may extend into the posterior chamber of the eye. The iris is frequently invaded, but extraocular extension rarely occurs in transplants of the first generation. The relatively small and confined space in the anterior chamber and the progressive increase in intraocular pressure with resulting circulatory disturbances seem to limit the size the tumor may attain. Once its nutritional requirements are exceeded it gradually undergoes fibrosis or regression. When the transplanted tumor extends into the vitreous, or is implanted there, the growth attains much greater mass.

1) HETEROTRANSPLANTATION OF FROG CARCINOMA. Transplantation to foreign hosts has been investigated (78) by planting the tumor into the anterior chamber of two other species, belonging to the same family of frogs (the green frog, *R. clamitans* and the bull-frog, *R. catesbeiana*), to a species belonging to a different family (the toad, *Bufo americanus*), and to animals belonging to different classes of cold-blooded vertebrates (goldfish, alligator). In the eye of frogs of alien species, the kidney carcinoma becomes as readily established as in the eye of the natural host. The rate at which growth progresses in these alien hosts is not notably different from that in leopard frogs. In toads, the proportion of successful transplants is somewhat less, but the character and rate of growth is practically the same as in the homologous species. It is possible to engraft the tumor, first, in the eye of the natural host and then to transplant it to the eye of an alien species, and finally to retransplant it to the original species. In this way the tumor has been propagated for 443 days. Similarly, tissue from leopard frog carcinoma grown *in vitro* grew progressively in the eye of a bull-frog. In contrast, no progressive growth of frog carcinoma occurred in fishes or reptiles, although in the former the implants survive for long periods, and a few mitoses, denoting continued proliferation, have been found 134 days after transplantation. In alligators, the transplants usually regress within 30 days. In both fishes and reptiles, the implants call forth a prompt exudative reaction and the aqueous humor becomes cloudy. Wandering cells accumulate around the tumor within 24 hours. It is not known whether the more rapid destruction of the amphibian tumors in alligators is to be attributed to a greater degree of specific incompatibility than exists in fish, or whether it is due to nonspecific factors, such as variation in osmotic pressure.

2) SERIAL INTRAOCULAR TRANSPLANTATION OF FROG CARCINOMA. The studies discussed above have been based largely upon intraocular transplants during their first generation, i.e., transplants taken directly from primary kidney tumors. In other experiments it was found possible to maintain the frog carcinoma successfully in the anterior chamber of the eye through 14 serial generations over a period of two years and three months (112). Repeated transfer and prolonged maintenance in the anterior chamber did not affect either rate of vascularization, rate of growth, or the size the tumors attained. It did, however, affect the incidence of successful transplants and their capacity to invade. In the first generation the tumor grew in only one of 10 frogs inoculated. When the successful transplant had filled the anterior chamber and bits of it were retransplanted, 4 out of 5 of these transplants became established and in the next generation 6 out of 7 grew. In the fourth gen-

eration the incidence of successful transplants rose to 100 per cent, and was maintained at this level during all succeeding generations.

The most conspicuous effect of serial transplantations was an increase in the ability of the growing tumor grafts to invade and destroy adjacent tissue. In the first few generations the tumors usually invade only the loose and spongy iris, whereas the hardy, unyielding cornea offers an effective barrier to invasion. Upon continued transfer, however, corneal invasion with erosion and extraocular extension of tumor became common. Thus, in the first 7 generations corneal invasion and perforation occurred irregularly and affected only 12.6 per cent of the animals, whereas in each of the following 7 generations it was frequently observed and the incidence rose to 44.8 per cent.

In another series of experiments the behavior of metastatic tumors was investigated by serial transplantation through 5 generations (385 days). It was thought that perhaps such tumors, having already established themselves in a tissue alien to that of their origin, would grow more luxuriantly than primary tumors. This did not prove to be the case. In the same environment, the aqueous humor, the growth of metastatic tumors was quite similar to that of primary tumors.

3) TRANSPLANTATION OF FROG CARCINOMA TO TADPOLES. Briggs (16, 18) has studied the behavior of this tumor by implanting it into various sites in tadpoles, that is to say, into an environment where the mechanisms for controlling growth and morphogenesis are more active than they are in the adult animal. He found that the larval environment neither inhibited the growth nor altered the structure of the implanted tumor. Successful transplantation was accomplished in approximately 23 per cent of 163 tadpoles. Minute implants often grew to large size in the sites selected, namely subcutaneous tissue of trunk, mesenchyme of dorsal tail fins, perirenal tissue, the liver, and the body cavity. It was not possible to observe the effects of metamorphosis of the tadpoles on the tumors in all of the implantation sites, for tadpoles with implants in the liver and body cavity were sacrificed earlier. But of 17 grafts in the subcutaneous tissue, 10 survived metamorphosis unaltered by this process.

The implants in the tail fin grew well in young tadpoles, but regressed rapidly as the tadpoles approached metamorphosis. Regression was associated with an accumulation around the tumors of spindle-shaped mesenchymal cells, and occasionally of small round cells. This cellular reaction was independent of the processes responsible for metamorphosis, for it was equally prominent in 'permanent' tadpoles, i.e., non-metamorphosing, thyroidectomized and hypophysectomized individuals. The mechanisms inciting the cellular reaction which seems to bring about regression are as yet unknown. Briggs observed two exceptional instances in which the carcinoma implants in the tail failed to regress and grew to relatively large sizes. One tumor (which is shown in photographs) grew steadily in a hypophysectomized tadpole, and had increased approximately 110,000 times in mass before the death of the host. The other persistent implant was in a normal tadpole. The tumor survived metamorphosis and grew an additional 75 days, when the frog died. This latter case is of particular interest as it supplies additional evidence in support of the belief that factors which bring about metamorphic absorption of the tail tissues exert no deleterious influence on the implanted tumors.

Studies such as those of Briggs may be expected to give valuable information on the cellular factors concerned in the regression of tumors.

4) TRANSPLANTATION OF FROG CARCINOMA TO NEWTS. Rose and Wallingford (104) have reported successful transplantation of the frog carcinoma to the forelimb of the newt, *Triturus viridescens*. Upon amputation of the limb through the implanted tumors, the authors observed changes which they interpret as differentiation of neoplastic cells to normal cells. If these experiments were confirmed they would be of great significance. Our own attempts to transplant frog tumors to newts have been unsuccessful.

c. *Tumors in reptiles.* Few attempts have been made to transplant reptilian tumors; all have been unsuccessful, perhaps because of inadequate methods or unsuitable hosts. We ourselves have tried to transplant a carcinoma of a large lizard, *Tupinambis nigropunctatus* and melanomas of two pythons, *Python reticulatus*. The lizard tumor was a squamous cell carcinoma which arose in the buccal cavity. Approximately one year later a similar growth developed nearby. Both tumors were repeatedly excised but promptly recurred. Since no lizards of this variety were available, bits of the tumors were transplanted to the gums of four alligators and to the subcutaneous tissue of 23 lizards of 3 different varieties. None of the grafts grew. Similar negative results followed attempted transplantation into the anterior chamber of the eye of 9 alligators, 6 chicks, 12 adult chickens and a number of other animals.

The first of the reptilian melanomas occurred in a huge python which had a large rapidly growing tumor on the lateral surface of the body. The growth became ulcerated and the animal was destroyed. At autopsy several metastases were found in the internal organs. In view of the successful transplantation of mammalian tumors to the yolk sac of chick embryos, it was thought possible to use this technique for the reptilian tumor (123). The finely minced melanoma was inoculated into the yolk sac or onto the chorio-allantoic membrane of 80 chick embryos. All died within 48 hours after inoculation. In some, death was the result of bacterial infection, in others no infection was present but the snake tissue appeared to be toxic to the embryo. The second melanoma which we attempted to transplant occurred as multiple primary growths in the same species of python, and probably had not attained malignancy. Intraocular transplants were made in 7 alligators, 1 turtle, and 18 frogs. None of the grafts became established and all were resorbed within 3 to 4 months.

### *Explantation*

It is generally accepted that neoplasms can be more readily grown *in vitro* than their homologous normal adult tissues. The two tumors of cold-blooded vertebrates which have been studied successfully in tissue culture conform to this rule.

1. *Melanoma of Hybrid Killifishes.* Grand, Gordon and Cameron (44) have shown that these tumors grow readily in a mixture of chicken-plasma, fish-serum and chick embryo extract. In the early stage of melanosis, outgrowths from explants are scanty, whereas cultures of fully developed tumors grow luxuriantly. The preponderant and characteristic cell type are the melanoblasts and in some cultures they are almost the only kind of cell present. Melanophores are numerous only in cultures

of explants obtained during the early stages of melanosis. In cultures of melanotic tumors, the dominant cells are melanoblasts. Except for the occasional presence of melanophores, the cell types appearing in tissue culture of fish melanomas are similar to those from human or mouse melanomas. In all 3 species the predominant cell, the melanoblast, is similar in its cultural characteristics. In view of the disputed histogenesis of melanomas it is especially noteworthy that in all 3 species, cells which have the characteristics of epithelium are never observed in tissue culture (43).

2. *Carcinoma of the Leopard Frog.* The renal adenocarcinoma of the leopard frog may be cultured readily in media containing diluted chicken plasma and frog plasma (74). In comparison with their homologues, the carcinoma cells are considerably larger and more variable. Their cytoplasm is less transparent, somewhat dense, and more apt to contain an abundance of fine granules. The nucleus is usually more sharply outlined, larger and less uniform in size than in normal cells. The nucleoplasm is so coarsely granular as to suggest early prophase of mitosis. The nucleoli are conspicuous and may attain very large size. Mitochondria are abundant, and radiate as long coarse filaments from the central region to the periphery. When cells are viewed under favorable conditions, a conspicuous centrosphere may be seen. The centrosphere and the adjacent nucleus are surrounded by a ring of granules and fine fat droplets. The number of mitoses observed varies in cultures from the same tumor, possible because of the periodic occurrence of cell division. The chromosomes, as in amphibian cells generally, are large, and where the number can be counted, 26 are found.

The cells growing out from the explants do not develop intranuclear inclusions, even though the latter may be abundant and persist in the original fragment used for explantation.

At the margin of the outgrowths, cells often become detached from their fellows, and exhibit active, though slow and ponderous locomotion, by means of broad ruffled pseudopodia. The rate of locomotion is accelerated by an increase in environmental temperature.

The various characteristics remain unaltered in subcultures maintained for as long as 6 months, i.e., the frog carcinoma cells, like malignant mammalian cells, are permanently altered and do not revert to normal type during cultivation.

The frog carcinoma is a transmissible disease due to an agent which induces inclusion bodies, and which has other attributes indicating that it is a virus. The general correspondence in character between its cells and malignant cells of mammalian tumors of diverse origin suggest that neoplastic phenomena are essentially alike no matter in what group of animals they occur or what their causal factors may be. The large size of cells, and the prominence of the intracellular structures, make this tumor favorable material for cytological studies.

### *Morphogenesis*

The characteristics of cancer growth have hitherto been studied chiefly by histologic methods, that is in fixed, sectioned, and stained material. But neoplastic growth, like normal growth, is dynamic, not static. Neither the dynamics nor the

mechanics of growth can be investigated adequately by histologic methods. Rather, growth should be studied by techniques that permit continued observation of the living tumor. By transplanting small bits of tumors into the anterior chamber of the eye and observing them with the slit-lamp microscope, or a similar device, the manner of growth of living cells may be observed by direct examination. The carcinoma of the kidney of the leopard frog has proven particularly suitable for this purpose. In the anterior chamber, details of form in the growing tumor can be observed with ease, and can be recorded by photographs from the earliest to the most advanced stages (77).

1. *Manner of Growth of Intraocular Transplants of Frog Carcinoma.* It will be recalled that the anterior chamber is a cavity filled with a transparent fluid, the aqueous humor, and bounded by tissues some of which are firm and relatively unyielding, cornea and lens, whereas the iris is soft and spongy. The bits of tumors implanted into this chamber were from large invasive adenocarcinomas; they invariably developed according to definite and well-defined growth patterns, their type depending on the immediate physical environment. Three such morphogenetic patterns were recognized (77).

If the outgrowths from the cancer extended into the anterior chamber of the eye where they were completely surrounded by fluid medium and unimpeded by solid tissue, the cells formed tubular or cylindrical structures. First, small hemispherical buds of cells formed at the free surface of the transplant; these buds gradually elongated to solid, straight, later slightly bent, cylinders with bulbous ends. With elongation, most of them acquired a central lumen containing fluid. The cylinders which now had become tubules, with still further accumulation of fluid, became cystic. They increased in complexity by repeated dichotomous branching and sac-like outpouching. In the longer tubules, local dilatations alternated with constrictions which gave them an unduloid shape.

If no lumen was acquired, a fibrovascular stalk gradually pushed into the cylindrical cell mass, which thus became a papillary projection. Such stalks were always derived from the tissue of the host and usually remained quite delicate, each consisting of a thin-walled vessel with very scanty stromal support.

The cylindrical form of growth persisted unless the proliferating cells made contact with a firm surface. When that happened, a striking change took place; the outgrowths adhered to the solid tissue and spread over the supporting surface as sheets of cells, that is to say, they now formed membranes instead of tubules. A membranous growth developed under two conditions: when the tip or margin of a free projection reached the lens or the cornea, or when the cells made contact with solid surfaces as soon as outgrowth began. Once membranous growth had started, it tended to spread laterally as long as the creeping cells met no obstacle. Where the cells had proliferated excessively or where they encountered irregularities in the surface, they became heaped up and protruded into the cavity as new cylinders.

A third type of growth was observed when the proliferating cell mass, either membranous or cylindrical, pushed into the loose tissue of the iris. Then the tumor reverted to the adenocarcinomatous form which it had in the kidney. Within the clefts of the tissue the invading cells became arranged as spheres or cylinders, usually

with a definite lumen, and always supported externally by a stroma. Invasion of the tissue also brought about a change in cellular polarity, for cell surfaces formerly directed outward now were turned inward. This change in polarity may be likened to turning the fingers of a glove inside out.

Thus it may be concluded that the form assumed by growing tumors is greatly influenced by the structures with which they come in contact. Evidently neoplasms are more responsible to laws that govern growth and organization than is generally recognized. These investigations of the growth patterns of living tumors support conclusions drawn from studies of human and other mammalian tumors based on histologic and reconstruction methods; references to the pertinent literature are given by Foulds (33). In seeking an explanation for the observed facts, it appears very probable that the factors that determine the patterns of growth are mainly surface forces. There is no doubt, however, that other factors, genetic as well as physico-chemical, collaborate in determining form (75, 128, 131).

2. *Manner of Growth of Frog Carcinoma in Tissue Culture.* Another method which permits further analysis of the mechanism of growth is tissue culture, which has much in common with the method of transplantation to the anterior chamber of the eye. In both methods, tissue is placed in a clear medium which is confined within a transparent chamber; nutrition and metabolic exchange of the implant are carried out, during the earlier stages at least, solely by diffusion; that is to say, without a blood supply; both methods permit direct and continuous observation of living tissue, but each has certain advantages which render it complementary to the other.

As already stated, this carcinoma may be cultivated in a plasma medium (74). Within a day, bud-like projections form and promptly grow out into the medium: they are sufficiently translucent to show details of component cells. With further growth, the projections elongate and acquire definite cylindrical shape. Lumens are not usually formed, nor do the cylinders ever acquire a fibrous core. The length which the outgrowths attain varies considerably, but many cylinders extend for a distance of about 5 times their diameter. When the growing cylinders make contact with the surface of the glass the character of growth changes entirely. At the point of contact the hitherto sharp contours of the cylinders become lost, and, within a few hours, proliferating cells burst forth and spread out over the surface of the glass as thin fan-shaped membranes. These gradually fuse with similar outgrowths until the explant is completely surrounded by a flat membrane. Where the margin of the explant comes in immediate contact with glass, the outgrowths are membranous from the beginning. Further growth is largely a repetition of the events described. From the periphery new cylinders continue to grow out into the plasma, but the cylindrical shape is always lost and membranes are always formed where contact is made with a solid surface.

The manner of growth *in vitro* is found to resemble closely the growth of transplants in the anterior chamber of the living eye, thus suggesting that in each instance the form which growth assumes is determined by the same morphogenetic factors, i.e., those inherent in the cells themselves and those largely dependent on the physical environment.

3. *Development of Apposition Tumors in Catfish.* Catfish with epithelioma of the lip or buccal mucosa develop a new tumor in direct apposition to the primary growths.

This occurs so frequently that it has been possible to study the beginning and further growth of these tumors by direct microscopic observation of the mucosal surfaces (80). The earliest visible evidence of neoplastic change is the establishment of a patch of intense hyperemia in that region of the mucosal surface which later becomes the site of epithelial proliferation. This occurs at a time when the mucosal surface is still entirely smooth. Approximately 2 weeks after the beginning of hyperemia, the mucosa begins to thicken and becomes slightly elevated. Within 1 or 2 months the local thickening progresses to the formation of a definite tumor.

Study of the hyperemic patch shows that profound alterations of the blood vessels accompany the development of the neoplasms. The vessels are greatly dilated and form an irregular wide-meshed anastomosing net, in sharp contrast to the uniform, small capillary loops of the adjacent normal mucosa. The caliber of the vessels in the neoplastic zone is variable and many have dilated bulbous expansions. The walls of the vascular channels are atypical, some are very thick, while others are extremely delicate. In spite of the abnormality of the vessels, blood flows through them swiftly. With the gradual thickening of the mucosa, the vascular pattern becomes obscured, preventing direct observation.

The relation of blood vessels to tumor growth is of importance but has not received much attention. As long ago as 1865 Thiersch (126), through injection of numerous human skin cancers, recognized that the vascularity of the early tumors is greatly increased over that of normal skin. Later, Boll (10), from a study of early epitheliomas and of the growing edges of such tumors, concluded that changes in the blood vessels determine the proliferation of the epithelium. In experimental tumors, Goldmann (38) noted profound vascular reactions at the onset of epithelial proliferation. Shope (116) reported that in infectious papillomatosis of rabbits, the subcutaneous tissue underlying the warts exhibits an enormously increased blood supply. Pertinent to this subject is the marked hyperemia which develops in a localized area on the floor of the mouth of brook trout as the first macroscopic evidence of hyperplasia or 'carcinoma' of the thyroid (36, 86). For other studies on vascular responses to tumors, see the following references: 1, 2, 24, 55.

The significance of the vascular changes which precede neoplastic growth has not as yet been elucidated. The catfish epithelioma appears to be favorable material for such investigation in the living animal.

### *Effect of Temperature*

It is an established fact that normal growth and development are influenced by temperature not only quantitatively but qualitatively (6, 128, 131). While the most obvious effect is on rate of growth, temperature also has a differential effect, for temperature does not influence all processes alike. Some may be accelerated, others retarded.

For studying the effects of temperature on cancer, the frog carcinoma is very suitable. Since the temperature of this animal is practically that of the environment (as was repeatedly demonstrated by thermocouple measurements) we have an opportunity to investigate the effects of temperature on neoplasia over a far wider range than would be possible in warm-blooded animals.

#### *1. Effect of Temperature on Manner and Rate of Growth of Intraocular Tumor*



*Transplants.* Several related groups of experiments were designed in which the influence of temperature was investigated by direct microscopic examination of intraocular transplants (79).

In the first series of experiments, the frogs, immediately after inoculation, were transferred to a constant temperature room at  $7^{\circ}\text{C}.$ , and compared with groups kept in the vivarium at  $22^{\circ}\text{C}.$ ; that is to say, at approximately room temperature. They were observed for periods of 2 to 9 months. It was found that tumors were able to establish themselves and grow at both temperatures. The main difference was in the rate of growth, this being much slower at the lower temperature. At  $7^{\circ}$  there was slow but continued outgrowth of cylinders or tubules which usually remained short and stubby and the size of the transplants increased very gradually. In contrast, at  $22^{\circ}$  growth was more rapid and the size attained was much larger. A further difference was the earlier and more effective vascularization of the implanted tumors in the group at  $22^{\circ}$ ; this, in turn, was associated with more massive tumor growth.

In the second group of experiments, the effect of much higher temperature,  $28^{\circ}\text{C}.$ , was compared with the low temperature of  $7^{\circ}$ . Now, certain effects of temperature became strikingly evident. For example, at the higher temperature the tubular outgrowths frequently became dilated and formed cysts, whereas these were rarely found in the 'cold' group. In all groups, growth of tumors continued, again at a slower rate at the lower temperature. But, though the initial rate of growth was greater at the higher temperature, growth retarding factors also came into play much sooner. Regression of tumors occurred in a higher proportion at  $28^{\circ}\text{C}.$  than at the lower temperature.

In the next group, the effects of temperature upon well established, large, vascularized tumors were investigated. When frogs with such tumors were transferred from the vivarium to the cold room, the growth of tumors was also greatly retarded.

In a final group of experiments, the effects of natural hibernation were observed. After inoculation, frogs were transferred to suitable cages in an outdoor pool just before ice formed on the surface. The frogs remained submerged in a condition of hibernation at approximately  $4^{\circ}\text{C}.$  for 10 weeks during which the ice persisted. The frogs were then removed and examined. Practically all of the implanted tumors were found to be viable as evidenced by their translucent appearance, but there was no definite evidence of growth. The animals were then placed in the vivarium at  $20^{\circ}$ , at which temperature active growth soon began and continued at the usual rate.

The most striking effect observed in these experiments is the acceleration in the rate of growth of the tumor at higher temperature, and retardation at lower. The ultimate size attained within periods averaging 3 months is regularly much greater at higher temperature. Also, the character of growth is quite different. At high temperature, there is a greater vascularization, and the tumors form long, branching, tubular outgrowths and cysts, whereas at low temperature the outgrowths are short and stubby and cysts are rare.

*2. Induction of Metastasis of Frog Carcinoma by Increase in Environmental Temperature.* Metastasis signifies the successful transplantation within the host,

by 'natural' means, of bits of detached tumor to tissues or organs alien to the site of origin. Information concerning the mechanisms concerned in this process is meager (132). It is known that at least 3 steps are involved in the formation of a metastatic tumor: initially, invasion of tissues (including vascular channels and body cavities); next, transport of detached bits, and their mechanical arrest; finally, growth of these arrested fragments and acquisition of a vascular stroma. In frog carcinoma, some of these sequential events are profoundly affected by temperature. It has been shown, first, that invasiveness of intraocular transplants becomes enhanced with increase of environmental temperature (79). Invasiveness itself, according to studies by Coman (23), and by McCutcheon, Coman and Moore (83), depends to a considerable extent upon decreased mutual adhesiveness of malignant cells, thus facilitating their detachment from each other, whereby they are enabled to wander into the surrounding tissue. In tissue cultures of frog carcinoma, elevation of temperature causes not only more ready detachment of cells from the margin of the outgrowth, but an increased rate of locomotion of the separated cells (82). Hence, the likelihood of tumor cells entering preformed channels in which they may be passively transported is increased. But such emboli do not of themselves constitute metastases, since most of them are probably killed (82, 132, 133). In order to form secondary tumors there must take place growth of the tumor cells, invasion of adjacent tissue (which itself must afford a suitable soil), and acquisition of a vascular supply. All these events appear to be favored by increase of temperature.

It seemed likely, therefore, that metastasis of frog carcinoma might be induced by increasing the environmental temperature for a sufficient length of time. This proved to be the case (82). When tumor-bearing frogs were maintained for approximately 50 days at a constant temperature of 28°, metastatic dissemination developed in 54 per cent of the animals, whereas only 6 per cent of frogs kept at 7° or 18° were found to have secondary growths. Moreover, at elevated temperatures the metastases were usually more numerous and more widely disseminated. They were also of fairly uniform size, suggesting that they had developed at nearly the same time. In some animals, the liver and lung particularly were so riddled with secondary tumors that the organs were almost completely replaced. Such extensive involvement has seldom been encountered by us in recently caught frogs, or in frogs maintained under fairly natural conditions of environmental temperatures. Dissemination of the tumors was influenced by the nutritional state of the frogs, occurring more readily in well nourished than in poorly nourished animals. This fact suggests that host factors play an important part in the evolution of a metastatic tumor.

While temperature profoundly affected the process of metastasis, under the conditions of the experiment, there was no significant or uniform effect on growth of the primary tumors in the kidney as was shown by periodic Roentgen-ray examination. No correlation was found between change in size of the kidney tumors and the incidence of their metastases. Metastasis occurred, or failed to occur, irrespective of whether the primary tumor became larger, remained stationary, or decreased in size. From these experiments it must not be concluded that temperature is the only factor concerned in the process of metastasis of a cancer in a cold-blooded animal; doubtless a number of other factors operate.

### *Induction of Neoplasia*

1. *Attempts to Induce Neoplasia by Means of Chemical Agents.* The relatively few experiments reported in the literature are limited to one species of fish and to several species of amphibians. No report has been found of an attempt to induce tumors in reptiles.

In fish, Li and Baldwin (67) reported the development of interstitial cell tumors of the testicle in 3 out of 40 adult swordtails, *Xiphophorus hellerii*, after repeated retroperitoneal injections of sesame oil. In two of the fishes the tumors were regarded as malignant because of their local invasiveness and the destruction of the surrounding gonadal tissue. In the third fish, small nodules composed of interstitial cells were present, but these nodules were thought to represent a hyperplastic rather than a neoplastic process. Further work is required before the nature of these testicular tumors can properly be evaluated. It should be pointed out that sesame oil, especially if overheated, has been found to be carcinogenic for mice (121).

In amphibians one of the earliest experiments is that of Champy and Vasilu (21) who attempted without success to induce tumors in the newt (*Triton cristatus*) by painting the skin with tar. Similar experiments by Martella (84) using the same species were likewise unsuccessful; ulcers and cachexia developed but there was no sign of neoplastic growth. More recently Duran-Reynals (27) injected dibenzanthracene (dissolved in lard) in the muscles of 18 newts (*Triturus pyrrhogaster* and *Triturus viridens*). Of these only 4 survived for more than 2 months; none of them developed tumors. Koch, Schreiber and Schreiber (61) have claimed some success after repeated subcutaneous injection of tar or of benzpyrene (dissolved in olive oil) into the 31 newts (*T. cristatus* and *T. taeniatatus*). They observed proliferation of cells around the injection sites, epithelial hyperplasia, and in one animal, nodules in the heart and lung. No satisfactory evidence for actual neoplastic development is presented. Hellmich (53) injected Sudan III subcutaneously into a salamander, *Amblystoma tigrinum* and 3 newts, *T. cristatus*, and reported the development of a 'preblastomatous process' in one of the newts. Russian workers have been most active in this field of investigation. The pertinent literature is given in a recent paper by Finkelstein (32).<sup>5</sup> In his own experiments, 200 axolotls received 3 subcutaneous injections of methylcholanthrene (in sunflower-seed oil) in the hind leg or in the dorsal fin. With this carcinogen Finkelstein had obtained various kinds of malignant growths in mice. The results in the axolotls were entirely different. After a period of 1 year, only 4 of the animals exhibited some type of proliferative reaction, namely, intense multiplication of endothelial cells in 3, and marked proliferation of the germinal layer of the epiderm in 2 of the axolotls. The proliferating epithelial cells permeated the corium and the tissues of the fins. But the hyperplastic cells were not atypical either in structure or in the character of their mitoses, and there was no destruction of the invaded fin. Finkelstein remarks that his results were similar to those of Fedotov who injected a number of axolotls subcutaneously with 1:2:5:6 dibenzanthracene and 9:10 dimethyl-1:2 benzanthracene. Only proliferation of the epiderm resulted, similar to that which occurred after the

<sup>5</sup> We owe the translation of this paper and of Schroeders' article (113) to the courtesy of the Army Medical Library.

introduction of cartilage, or of the products of its hydrolysis. It is very doubtful, therefore, whether the proliferative changes observed can be considered neoplastic.

More difficult to interpret are the experiments reported by Shevchenko (115), who introduced crystals of methylcholanthrene into the leg muscles of axolotls. Shortly afterward the crystals were surrounded by foci of proliferating fibroblasts. Growth was slow, but finally the proliferating cell mass invaded muscles and bone. The author regarded this as evidence of neoplastic transformation, and termed the lesion 'spindle-cell sarcoma'.

Of considerable interest is the report of Tokin (129), who achieved inhibition of regeneration by a carcinogenic substance. In his experiments he amputated the tarsus of 6 axolotls and 2 *Amblystoma*, and applied a watery solution of tar to the stumps. These results, if confirmed, are of importance; they apply to a field of research in which amphibians offer many advantages. In connection with Tokin's work, the papers by Haddow and others (4, 47, 48, 50, 51, 120) on growth inhibition by carcinogenic agents should be consulted. Experiments of this nature have led Haddow to the formulation of an interesting hypothesis concerning the nature of neoplasia.

Turning now to experiments on anura (tailless amphibians), Duran-Reynals (27) injected the thigh muscles of 261 frogs belonging to 3 species with varying amounts of dibenzanthracene, benzpyrene and methylcholanthrene (in lard or in colloidal suspension). The animals were maintained under different conditions of temperature. All but 7 died within 4 months after injection. None of the frogs developed tumors at the site of injection. Since leopard frogs, the species chiefly employed in his experiments, are frequently afflicted with a renal carcinoma which is probably caused by a virus, special attention was paid to the incidence of this tumor because of the possibility that the carcinogens might activate a dormant virus. However, no difference in the incidence of kidney tumor was found between injected and control frogs. As Duran-Reynals points out, no conclusion with respect to the effect of carcinogen on virus can be drawn from these studies, for the substances used, though actively carcinogenic in some mammals, were found to be inactive in frogs.

Recently, Schlumberger (110) implanted crystals of methylcholanthrene directly into the kidney of leopard frogs. The experiments had two aims; the first was to determine the effect of this substance when brought into intimate contact with a tissue which is known frequently to be the site of a carcinoma. The results indicated that even under these conditions the tissues of the frog fail to become neoplastic. The second aim was to determine whether methylcholanthrene might activate a carcinogenic virus in the kidney. No evidence of such activation was found and for the reasons given above no valid conclusions can be reached. (For an example of interaction between a carcinogenic virus and a chemical carcinogen, see the paper by Rous and Kidd (107).)

Other experiments with negative results are those by Skapier (118) who attempted to induce carcinoma in European and North African toads by daily application to the skin of benzpyrene or methylcholanthrene (in oil). One hundred toads were used. All died within 15 to 25 days after the beginning of the experiments and

therefore no conclusions can be drawn. Kinoshita (60) also employed toads for intramuscular injection of several carcinogens and no tumors developed.

Briggs (14) introduced methylcholanthrene choleic acid into 154 young tadpoles. Of these only 12 retained the injected substance and in 3 of these a connective-tissue mass formed which Briggs regarded as a tumor. The same author tested the effect of a water-soluble carcinogen on early frog development. Eggs of *R. pipiens* were reared in a solution of this compound, the exposure beginning one hour after fertilization. There was no evidence of a stimulating effect in any of the several concentrations used, but on the contrary, in the stronger concentrations the rate of development was retarded after the beginning of gastrulation. The exposure to the chemical had no effect on organization of the embryo, nor did any tumors arise (13).

The various experiments reviewed above lead to the following conclusions. No convincing evidence has been produced that true neoplasia has been induced by any of the chemicals used in any cold-blooded vertebrate. It is therefore not possible to answer the question which has been raised by Needham (91), and others: "Is there any difference in susceptibility to cancer between urodeles which can regenerate lost limbs and tails so well, and anura and reptiles which have no such capacity?" Probably most of the 'tumors' or proliferations noted can best be interpreted as granulomatous masses or as non-neoplastic hyperplasia resulting from prolonged irritation. But it is not possible to state categorically that cold-blooded animals are refractory to carcinogenesis by substances which in some mammals readily induce tumors. In many of the experiments cited, the time between the introduction of the chemical agent and the examination of the animal was entirely insufficient to bring about neoplastic change. Moreover, no chemical has a carcinogenic effect in all species; it may be active in one and inactive in another closely related species of animal. Even in the same species success depends upon a great number of variables. The writers of this review believe that the possibility of inducing neoplasia in cold-blooded vertebrates by chemical agents has not been completely explored. In view of the long latent period required for the development of tumors, often one-sixth to one-third of the normal life-span of the animal, it is of particular importance in future experiments to employ species which have a relatively short life-span.

2. *Attempts to Induce Neoplasia by Means of Embryonal Tissues.* Stimulated by Cohnheim's hypothesis (22) that embryonic rests may become neoplastic growths, many early investigators tried to produce tumors by injecting fetal tissues into adult animals.<sup>6</sup> Among the first to use cold-blooded vertebrates for this purpose were Birch-Hirschfeld and Garten (9) who in 1899 injected young salamander larvae into four adults. Except for a bit of cartilage found at one site of inoculation the transplants were wholly resorbed. The same procedure was carried out using tadpoles and adult frogs. One of the latter was subsequently found to bear a 1-2 mm. nodule of cartilage and a small cyst surrounded by giant cells at the site of implantation in the liver. A few tiny nodules of cartilage were also present in the lungs.

In 1906, Rössle (105) ground up three-week old trout embryos and injected them into the coelomic cavity of a carp and into the pericardial sac and peritoneum of

<sup>6</sup> For present day views regarding Cohnheim's hypothesis, see Willis (133).

four adult trout. None of the implants grew. Subsequently Gargano (35) working with elasmobranch fishes implanted embryos of the dogfish (*Scyllium stellare*) into adults of another species (*S. canicula*) and several rays (*Torpedo ocellata* and *T. marmorata*). He employed a total of 80 adults as hosts. The sites selected for inoculation were the subcutaneous tissue, coelom, spleen, or liver. All implants were rapidly resorbed.

Renewed interest was aroused in these studies by the report of Belogolowy (7) that he had been successful in producing sarcomas by homo- and heterotransplantation of the blastulae and gastrulae of the frog *Rana fusca* and the spadefoot toad *Pelobates fuscus*. Not only were 'sarcomas' observed following transplantations between these two anuran species, but even when larvae of the latter were placed in the coelomic cavity of salamanders or carp such tumors developed. The neoplasms arose in the connective tissue surrounding nodules of cartilage or cysts lined by stratified epithelium that represented the farthest stage of development reached by the transplanted embryonic tissue. These experiments were repeated by several investigators (3, 8, 96, 125) all of whom failed to observe the invasive growth reported by Belogolowy and interpreted his findings as examples of foreign-body granulomas induced by the presence of the embryonic tissue.

A number of investigators (references by Briggs, 15) have studied the abnormalities, including 'tumor-like' growths, which occur in the course of development of over-ripe frogs' eggs (i.e., eggs which have remained in the uterus for periods ranging from 3 to 5 days before they are fertilized). Such eggs gradually lose the capacity to develop into normal embryos. Together with a tendency to form monstrosities of various kinds, the embryos are said to exhibit neoplastic growths. Thus Witschi (134) states: "The epiderm is thickened, consists of much enlarged cuboid cells, and often by a wild growth gives rise to a characteristic epithelioma." He attempted transplantation of abnormally developed tissues to normal tadpoles and frogs. Most of the grafts became successfully established, and some progressed to masses which the author regards as malignant neoplasms. The evidence presented is not convincing.

Briggs (15) described benign papilloma-like ectodermal growths in the small proportion of embryos which developed from over-ripe eggs of leopard frogs. When transplanted to the anterior chamber of adult frogs or to subcutaneous tissue of tadpoles these growths regressed. Other transplantation experiments led to an interpretation of the probable nature of the "papillomas" (17). It was possible to show by measurement that the growth rate of over-ripe embryos bearing these structures was significantly retarded, whereas the papillomas themselves grew at approximately the same or at a slightly slower rate than the comparable ectoderm of normal embryos. The papilloma may therefore be regarded as an island of ectoderm growing at a normal rate on a tadpole which is growing at an abnormally slow rate. If this interpretation is correct, the papillomatous tissue should behave like normal tissue when grafted to normal hosts. This proved to be the case. When transplanted to the flank of normal tadpoles the hitherto exuberantly growing tissue became incorporated as a normal part of the host embryo. It was therefore concluded that the character of the papillomatous tissue is not irreversibly altered, for when placed in normal surroundings it again reverted to ordinary ectoderm.

Transplantation of fetal tissues has also been employed to study the probable mode of origin of teratomas. (Teratomas are neoplasms composed of multiple tissues of kinds foreign to the part in which they arise. For discussion of such tumors, see Willis (133) and Schlumberger (109).) These tumors, with their strange disarray of organoid elements, had long been regarded by some investigators as distorted embryos. Jacques Loeb's (68) induction of parthenogenetic development of sea urchin eggs was widely regarded as lending support to the belief that teratomas arise by parthenogenesis of germ cells. Likewise it was thought that the injection of whole or parts of embryos should give rise to teratoid structures. Along with other laboratory animals, amphibians were used in these experiments. Josephy (58) injected ground-up larvae of the newts, *Triton palmatus* and *T. alpestris*, into the coelomic cavity of adults of the same species. When the animals were killed 30 to 50 days after inoculation 'teratoids' were found in nearly all of the hosts. Nodules up to 2 mm. in width were present, which on histological examination were found to contain many tissues: cartilage, muscle, blood vessels, epithelial elements that lined cysts, and nerve cells.

The most comprehensive study of this kind is that of Bosaeus (11), published as a monograph in 1926. Using the larvae of the toad, *Bufo vulgaris*, and the frog, *Rana temporaria*, he employed the adults of both species as hosts for homo- and heterotransplants. The larvae were intact when introduced into the hosts. The sites selected were the dorsal lymph sac, coelom, and ovaries. No differential effect on the growth of the implants in the various regions was noted. As heterotransplants Bosaeus injected 90 toad gastrulae and 100 newly hatched toad larvae into 19 adult frogs. Similarly, he inoculated 220 frog blastulae into 22 adult toads. The embryos soon became necrotic and were wholly resorbed. Granulation tissue containing occasional giant cells was present, but no malignant change in the stroma was observed. Homotransplants were carried out using 1424 frog embryos transferred to 169 adult frogs and 896 toad larvae inoculated into 92 adult toads. In this series the embryos were also often resorbed or completely necrotic. However, in about 100 hosts there was evidence of a 'take', with some further development of the implant. Subsequently the embryos, vascularized by the host, underwent complete disorganization and were converted from independent organisms into poorly differentiated polycystic structures.

Bosaeus believed that ovarian teratomas of man arise from a parthenogenetically developing ovum. He therefore carried out a series of experiments in which parthenogenetic larvae were returned to the dorsal lymph sac or ovary of the female as autotransplants. The frog eggs were stimulated to develop by pricking with a needle, the toad eggs were inseminated with irradiated frog sperm. A total of 550 embryos were obtained from 80 adult females into which they were reimplanted at different stages of development. In general the growth and further development of these emplants differed little from that of transplanted normal larvae. Unusual, however, was the complete lack of an inflammatory response on the part of the host. It is worthy of note that gonads or germ cells were not found among the transplanted larvae in any series of Bosaeus' experiments. The absence of germ cells in human teratomas is often regarded as a deficiency that rules out the possible origin of such tumors from ova by parthenogenesis.

By cultivating the blastulae of newts in 0.35 per cent Ringer's solution Holtfreter (54) induced partial exogastrulation. This resulted in the formation of a mass of mesentoderm attached to the caudal end of the hind gut of the larval newts. Within this mass there subsequently differentiated glandular structures, muscle, bone, and cartilage. Holtfreter believes the result to be comparable to one of the most common extra-gonadal teratomas of man: viz., that of the sacrum. This view cannot be accepted. Masses such as those produced by Holtfreter are perhaps similar to congenital malformations but not to teratomas, for they fail to exhibit unrestricted neoplastic growth. Indeed, in none of the experiments just reviewed do the embryonic-tissue transplants give evidence of the independent progressive growth characteristic of true neoplasms.

#### *Biochemical Studies.*

In his admirable monograph on the 'Biochemistry of Cancer' Greenstein (46) summarized the notable advances that have been made in this field. His opinion is: "It seems probable that a cancerous tissue can be described by a chemical pattern which is largely similar to that of nearly all other cancerous tissues regardless of their etiology, histogenesis, or even species wherein found." Because of the ubiquity of neoplasia among vertebrates of all classes, and the close similarity in histologic structure and biologic behavior of structurally comparable neoplastic growths, regardless of class or species, it is desirable to extend studies of tumors in cold-blooded animals to an investigation of their biochemical activities. The only report to the present is that of Young, Breedis and Lucké (135) who studied the histochemical pattern of alkaline and acid phosphatase in the kidney carcinoma of the leopard frog. These enzymes were chosen because they are known to be present in the normal kidney of the frog. It was the particular aim of the study to determine the activity of these enzymes in relation to the development and growth of the kidney tumor. Using a slightly modified method of Gomori (39), it was found that during the growth of the frog carcinoma, from minute localized nodules to large invasive and metastasizing tumors, profound changes occur in enzyme content of the neoplastic cells. As the tumors become larger and more aggressive, alkaline-phosphatase activity diminishes and in many of the larger tumors disappears entirely. In contrast, acid-phosphatase activity increases until this enzyme becomes very abundant in the growing tumor. Metastases from the primary tumor, occurring relatively late in the course of neoplastic development, had a pattern of phosphatase distribution similar to that of the larger tumors. These results led to the conclusion that the activity of both acid and alkaline phosphatase becomes greatly altered, the former notably increasing and the latter decreasing during the course of development of the frog carcinoma.

The common occurrence and availability of a variety of tumors in cold-blooded vertebrates permit more extensive investigation of their biochemical properties. Thus it may be hoped to broaden the scope of knowledge concerning the general characteristics of neoplasia.

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# TRANSPORT OF IONS ACROSS CELLULAR MEMBRANES<sup>1</sup>

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**T**HE ABILITY TO CONCENTRATE certain substances and to expel others seems to be present in all living cells. Indeed, with the possible exception of certain intracellular parasites, no organism could exist without being able to take up and excrete a number of substances so as to maintain the proper conditions for the processes connected with life.

Although most biologists probably have a rather clear idea of the meaning of 'transport' or 'active transport,' it may be useful to define the term in an unequivocal way. In accordance with Rosenberg (121), the term 'transport' or 'active transport' will be taken to mean a transfer of a substance against a chemical potential gradient. This definition is rather narrow; for instance, cases where a substance is found to pass faster along the concentration gradient than would be expected from simple physico-chemical considerations will not be included. The definition also excludes cases where a substance is concentrated in a cell by being bound to some cellular constituent as is the case with many dyes. As regards charged particles (ions) it is logical to restrict the term 'active transport' to cases where the transfer takes place from a lower to a higher electrochemical potential. The electrochemical potential difference for an ion species between two dilute solutions at the same pressure is equal to  $RT \ln \frac{c_1}{c_2} + RT \ln \frac{f_1}{f_2} + zF(\psi_1 - \psi_2)$  where  $c_1$  and  $c_2$  are the concentrations and  $f_1$  and  $f_2$  the activity coefficients of the ion in the two media,  $\psi_1 - \psi_2$  is the electrical potential difference between them,  $z$  is the valency of the ion,  $R$  is the gas constant and  $T$  the absolute temperature. As is well known, the activity coefficient of single ions cannot be measured; but in dilute solutions it is permissible to assume that the activity of an ion is only determined by its charge and the ionic strength of the solution, and so it is possible to compute the activity coefficient.

The measurement of the potential difference across living membranes involves the use of liquid junctions, and if the solutions on the two sides of the membrane are not infinitely dilute or identical there will be an error due to the liquid junction potentials. If the liquid junctions are made through saturated KCl bridges, the junction potentials are reduced to a very small value. Nevertheless, this error makes liquid junctions undesirable in very precise physico-chemical work; but in most ordinary biological and chemical work the error due to liquid junctions is tolerated; as is well known, the standard methods of  $pH$  and redox potential measurements make use of liquid junctions.

The direct measurement of electrochemical potential differences in biological systems using electrodes which are reversible for the ion in question, is as a rule not feasible. If, for instance, one wishes to use silver chloride electrodes to measure the electrochemical potential difference for Cl, the solutions have to be saturated with silver chloride; but silver ions, even in very high dilution, are extremely poisonous to most living cells, so that, while avoiding a comparatively small error due to liquid junctions one destroys the system under observation.

This means that we shall only speak of active transport when work has to be done to transfer the ion across the membrane, whether this work is used to overcome a potential difference, a concentration difference or a combination of both. This definition has the advantage that, at least theoretically, a distinction is made

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between transported and passive ions in cases of salt transport. Ions which cross a membrane due to electric attraction should not be regarded as being actively transported; the ions just move from a higher to a lower electrochemical potential although they may go from a lower to a higher concentration. Interest is thus focussed on the process of increasing the electrochemical potential of an ion at the expense of chemical energy derived from the metabolism. It may turn out later that the distinction between apparently transported and apparently passive ions is not justified. Nevertheless, the successful application in recent years of the Donnan principle on the distribution of ions between muscle and nerve cells and their surroundings (9, 132, 51) has given us hope that the multitude of different ion distributions in cells and organisms can be traced back to a finite number of more or less well defined transport processes involving a small number of ions.

Within the limits of a review article it would be impossible to discuss all recent papers with a bearing on active ion transport. In itself, however, our definition of active transport as a transport from lower to higher electrochemical potential suggests a limitation of the discussion to such systems where the definition can be applied. In other words, we shall have, primarily, to deal with biological systems where in the first place the concentrations of free ions on both sides of the membrane are known and where, secondly, the potential difference has been measured. Moreover we shall include cases where the direction of the electrochemical potential gradient can be estimated indirectly. These requirements, modest as they may appear, are only fulfilled in the case of a limited number of transporting organs, and mainly for monovalent inorganic ions. The transport of the divalent cations  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$  and  $\text{Fe}^{++}$  for example is hardly ripe for discussion, because these ions are largely present in complex form in the cells, the concentration of the free ions being low and mostly unknown. For similar reasons we cannot enter into a detailed discussion of the phosphate uptake in the cells. The concentration of free phosphate in the protoplasm is nearly always low relative to the concentration of phosphate esters, and there is always a risk of some labile phosphate ester being split during the extraction of the inorganic phosphate. Moreover phosphate may at least penetrate in three different forms namely as secondary and primary phosphate and as phosphoric acid. The subject of phosphate transport has quite recently been discussed by Sacks (126, 127) and Kamen and Spiegelman (65).

#### HYPOTHESES OF ACTIVE TRANSPORT OF IONS

Although the exact nature of the forces bringing about the transport of ions is not known, physico-chemical considerations show that, in many cases at least, the transport must involve chemical reactions between cell constituents and the transported ion. No other force possesses sufficient specificity to perform, for instance, the separation of Na and K ions from a mixture of these ions. This standpoint is clearly expressed by Spiegelman and Reiner (137). After having calculated the energy requirement for K accumulation and Na exclusion from a living cell they find that electric forces alone cannot account for the separation of K and Na whereas chemical reactions may easily supply the necessary energy. Recently Rosenberg (121) has given a theoretical treatment of the problem of active transport on a thermody-

namic basis. He also excludes all forces but the chemical potentials as responsible for the transport processes. In the case of phosphate transport, for example, it is easy to imagine a formation of a phosphate ester at one boundary of a cell membrane and the splitting of the ester at another point in the cell. As a matter of fact there is some evidence that phosphate is actually taken up in muscle cells by being esterified with glucose (126).

The transport and especially the separation of Na and K has given rise to much speculation on account of the apparent similarity between these ions. This similarity should not be overemphasized, however. In their well-known model experiments with two aqueous phases separated by a layer of guaiacol, Osterhaut *et al.* (103) found an accumulation of K as compared with Na, evidently due to the higher affinity of guaiacol for K. On the other hand, according to Hodgkin (51), the tendency of Na to combine with a lipid carrier might conceivably be greater than that of K because cations give rise to covalent linkages more readily when the atomic number is small (133).

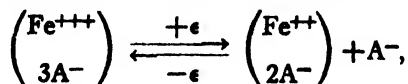
If we accept the view that a chemical reaction is necessary between the ion to be transported and some cell constituent the general picture of the system must evidently be the following: 1) a carrier forming a relatively stable compound (complex) with the ion and 2) a system which can react with the complex so as to set free the ion, either by changing the carrier chemically or by supplying another ion which can replace the transported ion in the ion-carrier complex (78, 51, 150).

In order that a net transport be brought about it is necessary that the formation and splitting of the complex are spatially separated. A theoretical treatment of some hypothetical systems of this type is given in a very interesting paper by Franck and Mayer (35). Their basic assumption is that a substance *a* is transformed into *b* in one end of a cell (the word cell is taken to mean a compartment which may be a cell in the morphological sense of the word or it may be part of a cell, for instance the cell membrane) whereas in the other end of the cell *a* is again formed from *b* by some chemical reaction. This system is clearly well suited to bring about a transport of *a*. The authors point out, however, that if the diffusion coefficients of *a* and *b* are different, depending on the permeability of the cell walls, the system may perform active transport of *a*, *b*, the solvent or any combination of them. The calculations show that under favorable conditions such a system may act as a very efficient pump for both dissolved substances and for water. This must remind us that even a system capable of doing water transport would be able to carry salts from one place to another. Such transport would, however, lack the specificity of true ion transport. The system described by Franck and Mayer (35) may in short be designated a solute circuit system because the water transport results when a solute is circulated in the cell. It may be of interest to compare this system with the formally related fluid circuit system which Ingraham, Peters and Visscher (63) proposed for the absorption of fluid and salt in the intestine. In this system it is the fluid which is assumed to be circulated so that a more concentrated solution goes in one direction and a less concentrated one is returned, thus bringing about a net transport of salt. In the original form of this hypothesis the difficulty of explaining salt transport was merely replaced by the difficulty of explaining transport of fluid. Later, however,

Ingraham and Visscher (62) suggested that the pumping action might be produced by anomalous osmosis (136) brought about by an electric current through the pores of the epithelial membrane. To bring about the electric current some ion must be produced at one end of the pore and be removed at the other end. The origin of the transporting force is evidently the same as in the Franck and Mayer pump, but the anomalous osmosis hypothesis makes use of the electric rather than of the chemical component of the electrochemical potential difference. The formation of organic ions or  $\text{NH}_3$  might conceivably produce the gradient required. Ingraham and Visscher (62) showed that  $\text{NH}_3$  is indeed concentrated in the gut as compared with the blood. But the relation between  $\text{NH}_3$  formed and salt absorbed is not a simple one and the authors do not consider it proven that the  $\text{NH}_3$  has anything to do with the transport.

Although watery solutions and organic matter are normally considered impermeable to electrons, there is at least a remote possibility that systems may be set up in living cells which will conduct electrons over short distances (143). This possibility serves as a basis for a transport hypothesis which has been advanced to explain the formation of the cerebrospinal fluid (142) and the intraocular fluid (37). Stiehler and Flexner (142) conclude from a comparison of the concentrations of different substances in blood and cerebrospinal fluid that this fluid is formed by a secretory process in the chorioid plexus. It was observed that in this organ basic dyes move from the stroma to the epithelium whereas acid dyes move in the opposite direction. This was taken to indicate that the movement of ions (and water as well) was brought about by an electric current. The source of the electromotive force for this current was supposed to be the difference in oxidation-reduction potential between stroma and epithelium. Since the potential level (as measured by oxidation-reduction indicators) of the stroma is lower than that of the epithelium, the former might give up electrons to the latter. The electrons were assumed to pass from stroma to epithelium by means of reversible oxidation-reduction systems contained in the stromal-epithelial barrier. To maintain electro-neutrality cations must then move from stroma to epithelium or anions in the reverse direction. It is difficult to understand that the alleged electric current brings about a Na concentration in the cerebrospinal fluid which is higher than that of the plasma, whereas the K concentration is only 60 per cent of that of the plasma. Actually, as shown by Greenberg *et al.* (39) K penetrates more easily into the cerebrospinal fluid than does Na. Although the electron transfer proposed in the hypothesis may play a part, specific transport mechanisms are probably involved. The Stiehler and Flexner hypothesis implies that the difference in oxidation-reduction potential can be identified with the electric potential difference between the two tissues. This has never been proved for any tissue and *a priori* it is not very likely.

Lundegårdh (94) has proposed an explanation of the uptake of anions in plant roots which is closely related to the Stiehler-Flexner hypothesis. Lundegårdh points out that the change of valency of the Fe ion in the hemin group of a respiratory enzyme is well suited to effect an anion transport according to the scheme



the trivalent Fe attracting one more anion than does the bivalent Fe. The hypothesis now assumes a series of these Fe-enzyme molecules arranged across a boundary between two media of different redox potential and "owing to the wavelike proceeding oscillation of the Fe valency they will transport anions from the medium with higher oxidation power to the medium with lower oxidation power." Whereas, granted the freedom of movement of electrons from one Fe to the other, such a system may transport anions, it will at the same time transport hydrogen ions. Every electron arriving at the oxidation side means one positive charge neutralized which is practically identical with the disappearance of one  $H^+$ . Similarly, the formation of the electron at the reduction side means the simultaneous formation of a hydrogen ion. Thus, what is transported is not simply anions as assumed in the hypothesis but anions plus hydrogen ions. Whether or not such a system will be suited to transport salts may ultimately depend on the mobilities of the different ions involved. A Teorell effect (147) due to back diffusion of  $H^+$  might conceivably result in the attraction of other cations. As a general explanation of transport processes these hypotheses share with other electric explanations the disadvantage of being unable to explain specific transport of one or few ion species. The specifically transported particles in the system just discussed are obviously the electron and the hydrogen ion.

#### DISCRIMINATION BETWEEN ACTIVE TRANSPORT AND DIFFUSION

Discussing the large differences in concentration of individual ions between the interior of living cells and the fluids surrounding them Krogh (80) writes: "... although permeability of the cell surface is of course a necessary corollary of the ion transport taking place, quantitative determinations of such permeabilities, in the general accepted sense of the term, can be made only by means of isotopes and even then require special precautions and conditions which are difficult to realize and verify, because the exchanges normally taking place are largely brought about by active transport." This statement rightly places much importance on the use of tracers in the study of the relation between transport and diffusion. As a matter of fact, whereas even the sensitive electrometric methods had failed to reveal any permeability to Na in most animal cells, the use of tracer Na has proven that the sodium ion can pass through practically all cell walls. But when one wants to evaluate this penetration in terms of active transport or diffusion he is up against considerable theoretical difficulty. The quantity which can be determined by adding a tracer at one side of a membrane and determining the rate at which tracer ions pass through may be designated the flux of the ion species in question (149, 150). The flux is defined as the amount of a substance which per unit time passes through unit area of the membrane in a given direction. If nothing else is known, however, a given ion flux may be the resultant of any combination of three processes, namely: *a*) free diffusion; *b*) exchange diffusion (see below); *c*) active transport. Although the present article should deal with point *c* only, a few remarks on the two types of diffusion might be justified because the active transport may be considered as the part of the flux which cannot be explained as diffusion. That cell walls generally are permeable to free ions is shown by the fact that they are conductors to a direct current, even though the resistance is very high as the excellent article by Cole (14) points out.



The theory of diffusion of ions through membranes and especially through living membranes is in a rather undeveloped state (23). Whatever the detailed mechanism is, it is evident that the flux in either direction must be the same when the ion in question is present at the same electro-chemical potential on both sides of the membrane. If the high K concentration in muscle and nerve cells as compared with the K concentration of the surroundings is due to a Donnan equilibrium then the K influx and the K outflux through the cell membranes must be the same and this flux value does not, of course, measure any active transport. If there is a difference in electro-chemical potential for any ion across a membrane, the influx and outflux will be different and the proportion between them will be a simple function of the difference between the electro-chemical potentials (see below).

The term exchange diffusion has been introduced to cover the phenomenon (83, 148) that an ion species crosses a membrane by combining with some carrier molecule which is part of the membrane or which cannot leave the membrane due to a selective solubility in the membrane phase. Due to the thermal movements the carrier-ion complex may alternately come into contact with either solution. When in contact with one of the solutions the complex may exchange the bound ion against one from that solution; but if the carrier-ion affinity is high, the carrier will always be saturated with the ion in question and the same number of ions will be carried from left to right as from right to left. The flux of the ion, as determined by the tracer method, would be nearly the same in both directions even if there were a considerable difference in electro-chemical potential for the ion across the membrane. The exchange diffusion thus simulates active transport and, as a matter of fact, both processes are mediated through carrier systems. They differ in that exchange diffusion does not require work and that it cannot bring about a net transport. On the contrary, there will always be some leakage through the exchange diffusion system because the affinity between ion and carrier is not infinite. The rôle of exchange diffusion for the exchange of substances across living membranes is at present unknown. We know that different molecules, having approximately the same size permeate the cell sometimes at very different rates. According to Reiner (119) this may indicate that many substances flow, not as free molecules but as adsorption compounds: solute—colloid. Reiner also gives a mathematical treatment of diffusion in such systems. The structure suggested for the cell membrane by Lundegårdh (88) would evidently lead to exchange diffusion. He assumes the membrane to be a Langmuir monolayer containing acid and basic groups which sometimes turn so that they are alternately in contact with both surfaces.

Brooks (10) believed that the rapid passing in and out of cations as well as anions in *Nitella* should be regarded as passive exchange. But his explanation differs somewhat from that given above. He assumed that the cell membrane possesses a mosaic structure so that some parts of the membrane were only permeable to anions and others only to cations. Such a membrane would, however, allow a leakage of positive and negative ions simultaneously and the system would hardly give any exchange-diffusion effect. Even though the explanation given by Brooks is not entirely correct the exchange in *Nitella* might well be due, in part at least, to exchange diffusion. The very possibility of this process at present limits the conclusions which

can be drawn from tracer experiments concerning the rate of active transport and of true diffusion. Used with proper care, however, the isotopes have provided a most valuable tool for the study of transport processes.

#### ACTIVE TRANSPORT OF CATIONS

*Does a K-transport Take Place in Animal Cells?* Whereas a simple analysis may show that the ionic composition of a cell is likely to be the result of active transport, it is far from easy to determine whether or not any specific ion species is being transported. A good example is found in the current discussion on the reasons for the high K content in most living cells. It is well known for instance that muscle cells are permeable to K ions. Most workers seem to be of the opinion that the greater part of the K in the cells is free (32, 33, 9, 80). This again implies that the K must either be transported actively into the cells or it must be held in equilibrium with the outside K due to electric attraction. In recent years the view has gained much ground that the cellular K not only of muscle but of animal cells in general stands in Donnan equilibrium with the outside K. The work of Conway and collaborators (9, 15, 16) has contributed much to our understanding of the ion distribution between cells and surroundings. In its original form the theory of Boyle and Conway (9) explained the distribution of K and Cl as the result of a double Donnan equilibrium, the system containing indiffusible anions (phosphate esters and proteins) inside the membrane and an indiffusible cation, Na, at a high concentration outside; K, H, Cl and a number of other small ions were free to diffuse in and out. The experiments of Fenn (33), Steinbach (140) and Heppel (45) show, however, that the muscle fiber membrane is permeable to Na. Indeed, in experiments with rats, having an increased Na content in the muscles as the result of feeding a low K diet to the animals, Heppel found that injected  $\text{Na}^{24}$  would equilibrate with the cellular Na within an hour, indicating a rapid Na exchange across the fiber membrane. Dean (27) therefore proposed to modify the Boyle-Conway theory by introducing the concept of a continuous extrusion of Na from the fibers by some pumping device. It is obvious that the distribution of passive ions would be the same whether the difference in Na concentration between cells and surroundings were maintained due to Na impermeability or due to a constantly working active transport. Krogh (80) also strongly advocated a rapid active elimination of Na from the fibers, a view based on calculations indicating that Na entered the fibers faster than K did. Conway (15) calculated the minimum energy required for the extrusion of sodium from the normal frog sartorius if sodium entered as fast as potassium. The result was that more than the energy available from the metabolism of the resting muscle would be needed, so that the permeability figures for Na must be too high. Nevertheless Conway now admits that an active extrusion of Na from the fibers must take place (16). The concept of exchange diffusion, in fact, bridges the gap between the views of Krogh and Conway because the part of the Na influx into the fiber which is due to exchange diffusion does not require active extrusion. Boyle and Conway (9) based their theory on experiments with isolated frog sartorii and although there was a very good agreement between theory and experiments at outside K concentrations exceeding about 10 mEq/l. the P.D. measured at physiological

K concentrations fell too low (at 5 mEqv. K/l the P.D. was 59.4 mV. against the calculated 78 mV.). This apparently means that the process responsible for the P.D. (Na extrusion from and formation of phosphate esters in the cells) does not suffice to keep back the K in the cells when the proportion between inside and outside K becomes too high. Graham and Gerard (38) using very fine capillary electrodes have measured the resting potential of single muscle fibers from frog sartorii. The values with normal K concentration in the outside solution average 62 mV. with some values as high as 80 mV. Although the high values would correspond to a Donnan equilibrium for K, most values are lower than corresponding to an equilibrium. This is, however, what could be expected if the fiber is slowly letting Na leak in while K is leaking out. It should be remembered that isolated muscles and fibers are not normal. It is therefore highly significant that Wilde (154, 155) has been able to verify Boyle and Conway's predictions in experiments on live rats. A stumbling block for the acceptance of Boyle and Conway's theory has been that it demands a Cl concentration of the fiber water of about the same magnitude as the K concentration of the blood plasma. The striated muscle fibers, however, were considered free from chloride (33). But recently Heilbrun and Hoagland (41) found that the standard methods for Cl analysis on muscle gave systematically low figures. Wilde (154) confirmed this and worked out a more reliable Cl determination. Using this method he succeeded in demonstrating a considerable difference between the Cl space of rat muscle on the one hand and the inulin and saccharose spaces on the other hand, indicating that part of the Cl had to be in the fiber water. Moreover there turned out to be a significant correlation between the concentrations of fiber chloride and plasma potassium. In order that the equilibrium condition  $(K_i)(Cl_i) = (K_o)(Cl_o)$  be fulfilled it was necessary, however, to assume the activity coefficient of fiber K to be only 0.55 indicating that part of the K in the fibers is bound. There is indeed reason to believe that part of the muscle K is bound more or less firmly. Some evidence was presented by Mullins (100) and Szent Gyorgyi (144) that the principal protein of the muscles, myosine, binds potassium specifically. Brues, Wesson and Cohn (11) following the exchange of K in cultures of embryonal muscle, found that the uptake curve for isotopic K could best be explained as the sum of three independent exchange rates which they tentatively considered to be: 1) exchange of interspace K, 2) exchange of free K in the fiber and 3) exchange of bound K in the fiber. Of course these findings might be explained differently. The same type of uptake curve would be achieved for instance if the fibers consisted of two phases with different permeability; yet the assumption that some of the K is bound might well be correct.

It thus appears that two factors contribute to bring about the high K concentration of the muscle fiber, namely the Donnan equilibrium and to a lesser degree the specific affinity between K and myosin. There is indeed little reason for assuming any active transport of K into the fibers.

A Donnan distribution of  $K^+$ ,  $Cl^-$ ,  $HCO_3^-$ ,  $H^+$ ,  $OH^-$  and other small ions seems to be realized in many animal tissues other than muscle. Shanes (132) working with spider crab nerves, found them to be freely permeable to KCl, but having a very low permeability to Na. At increased outside K concentrations the P.D. and the

ion distribution corresponded to the type of Donnan equilibrium described by Conway *et al.* for muscle. At low K concentrations, just as was the case with frog sartorii, there was a marked divergence between calculated and found P.D. values.

Hodgkin (51) found that small changes in external K concentration caused large and rapidly reversible changes in membrane conductance in the squid nerve fiber. Rb and Cs ions had a similar effect. Na and Li had to be added in 40 times higher molar concentration than K to bring about a similar effect. This must mean that the fiber membrane is much more permeable to free K ions than to free Na ions. (The low rate of Na penetration relative to the K penetration is also clearly seen in the tracer experiments performed by Rothenberg and Feld (123)). The fact that the outside concentration of K has such large effect on the membrane conductance was now used in an ingenious way to demonstrate that K is given off from nerves during activity and taken up again during recovery (52). During the experiments the nerve fibers were submerged in oil; the outside water phase was thus reduced to a thin film where the minute amounts of K leaving or entering the fiber could bring about measurable concentration changes. The reabsorption of K by the nerve fiber is probably of electrostatic nature, however, possibly as a result of the active Na extrusion.

At present there is little reason to assume any active K transport in nerve. This view is very clearly expressed by Hodgkin (*l.c.*): "It makes little difference to the potassium distribution whether Na is kept out by an active process or by a semi-permeable membrane. In either case the ratio of activity of potassium inside the membrane to that outside should be given by the expression  $e^{EF/RT}$ . The activity ratio could only exceed the value defined by the resting potential if active transfer of K took place. At present there is no clear evidence to show that the activity ratio does exceed this ratio, so that neglect of active transfer seems reasonable in a working hypothesis."

Although the necessary P.D. values are not available for other cell types with high K and low Cl concentration it seems at present reasonable to ascribe the ionic composition of these cells to a Donnan equilibrium of the type found in muscle and nerve. Indeed, clear-cut examples of true active transport of K in higher animals are few, up to the present. There is reason to believe that K can be absorbed in the gut of fishes to a lower concentration than that of the plasma (135). Likewise, a true K transport may perhaps take place in the kidney tubules. On the other hand Krogh was unable to find any K uptake in a number of fresh water animals (frog, goldfish and others) which take up Na eagerly even from exceedingly dilute solutions (79).

Certain crabs possess the ability to take up K as well as Na from the surrounding water. This has been demonstrated for the wool-handled crab, *Eriocheir sinensis* (79) and the common shore crab, *Carcinus maenas* (129). This cation uptake which does not discriminate between Na and K calls for further study. Especially, it would be interesting to know the direction and magnitude of the electric potential difference across the gill epithelium of these animals during active ion uptake. Until such determinations are available it is impossible to decide whether a true transport of K (and Na) is going on or whether the salt transport is really an anion transport.

*Potassium Transport in Plant Cells.* Although K as well as many other cations are accumulated in the protoplasm and the cell sap of most plants, it is by no means certain that the accumulation is generally due to active K transport. In *Nitella* and *Halicystis* the cell sap is negative relative to the outside solution under conditions similar to those prevailing during the uptake process. The material at hand does not make it clear, however, whether the P.D. always suffices to bring about the K accumulation. In root tips there is likewise a potential difference between the outside solution and the cut end of the root, which as to sign and magnitude would explain the transfer of cations from the medium to the bleeding sap (Lundegårdh (91)). It is important to note that the contact at the cut end of the root tip is made with the bleeding sap and not with the cell sap of the epidermal cells. Therefore we cannot tell whether the cation accumulation in the cell sap is of passive or active nature. On the other hand there is often a close correlation between the cation uptake of the whole root tip and the P.D. (130). The cation uptake in root cells will therefore tentatively be discussed under the heading anion transport.

There are, however, certain plant cells where a more or less specific K transport can be demonstrated. Pulver and Verzar (114, 115) found that yeast cells would take up K from the medium during fermentation and give up this K again at the end of the fermentation.<sup>2</sup> This K uptake has been studied more closely by Conway and O'Malley (19, 20, 16) and by Rothstein and Enns (122). It turned out that the K uptake consisted in an exchange of hydrogen ions against potassium ions. If the yeast cells were allowed to fermentate in an unbuffered solution containing KCl they would take up K and give up H until the  $pH$  of the medium reached about 1.6. If a similar experiment was made with NaCl in the medium instead of KCl, hardly any Na was taken up by the yeast; and although a  $H^+$  excretion was observed the  $pH$  of the medium was only lowered from 4 to about 3. In neither case was any Cl taken up by the yeast. At first Conway and O'Malley assumed the peculiar K-H exchange to be due to a Donnan equilibrium in analogy with conditions in animal cells. Later, however, they altered this view because the  $pH$  of the content of the yeast cells was found to be much higher (5.8–6.2 in fermentating yeast) than required by the Donnan equilibrium. The authors therefore offered the explanation that the cell content consisted of two regions of which the outer compartment, having a very acid reaction, was in Donnan equilibrium with the outside, whereas the rest of the cell was separated from the outer compartment by a special cation impermeable membrane. Such a system would lead to K accumulation only in the outer compartment. As this would hardly account for all cellular K, the authors suggest a mechanism actively transferring one of the ions, K or Na, across the boundary between the acidic region and the rest of the cell. As yet, the region with high acidity has not been demonstrated directly. Whereas the process bringing about the forced exchange of H against K is entirely unknown, Conway and Brady (17) have been able to demonstrate that the H ions excreted originate from succinic acid and acid-labile carbon dioxide. It was found that with one part of yeast to 0.6 parts of a

<sup>2</sup> A similar K uptake is also found in certain bacteria (84) and possibly in other micro-organisms (131).

5 per cent pure glucose solution, up to 0.2 per cent succinic acid was found in the suspending fluid. With KCl in the suspending fluid the amount of succinic acid found in the medium was much lower, but there was a corresponding accumulation of succinate along with the K in the cells. However, the succinic acid formed did not account for all the H given off, the remaining part being derived from carbonic acid.

Another spectacular case of K transport is found in the much studied marine alga *Valonia macrophysa*. Here the cell sap is positive (about 8 mV) relative to the sea water and despite this the K concentration of the cell sap is about 500 mEq/l against 12 mEq/l in the surrounding water. The Cl concentration of the sap is also higher than that of the surrounding medium (597 mEq/l against 580), but due to the P.D. the Cl might well be concentrated passively.

The rate of KCl transport into the sap of *Valonia* rises with increasing  $pH$ . This fact speaks in favor of Osterhout's assumption that the K crosses the cell membrane (and the vacuolar membrane) as a complex with some organic anion; a high  $pH$  favours the complex formation. A true active transport of K is obviously at work in *Valonia*. This view is expressed by Blinks (7) as follows: "Thus it appears to be the K ion which, if not responsible for the total P. D. of *Valonia*, nevertheless serves as the electrical indicator of various processes which are probably intimately connected with the accumulation of this element." The phosphate metabolism of *Valonia* seems to be very closely related to the K uptake. Thus Mullins (101) has shown that the rate of K accumulation increases when the external phosphate concentration is raised.

*Active Na Transport.* It has already been mentioned that an active extrusion of Na seems to be at work in most animal cells. In nearly all cells the Na concentration is lower than that of the tissue fluid. The use of tracers has given convincing evidence that Na can pass into nearly all cell types (45, 123, 95). Therefore, although it may be convenient, in electrophysiological considerations for example, to regard the cells as Na impermeable, this is not absolutely correct and the explanation of the function of the cell is incomplete if the effect of the Na transport is not taken into account.

For the following discussion of the Na transport it will be convenient to distinguish between cells where the transport serves mainly to maintain a more or less constant composition of the cell and such cells where a net transport takes place across the cell. The transport mechanism might well be the same in both cases; but the difficulties facing the experimenter are much greater in the first case.

The availability of isotopic tracers made it possible to determine the 'flux' of nearly all ion species across the cell membrane. In the case of muscle and nerve the part of the flux which is free diffusion can be shown to be very small indeed. Assuming the K ions to be distributed according to a Donnan equilibrium it can be shown (16, 83) that

$$\frac{M_{Na(out)}}{M_{Na(in)}} = \frac{C_{Na(l)} \cdot C_{K(o)}}{C_{Na(o)} \cdot C_{K(l)}}, \quad \text{where } M_{Na(out)}$$

and  $M_{Na(in)}$  denote the diffusion flux of Na outwards and inwards, respectively, and  $C_{Na(l)}$ ,  $C_{Na(o)}$ ,  $C_{K(l)}$  and  $C_{K(o)}$  denote the Na and K concentrations. The activity

coefficient for the monovalent ions is assumed to be the same within the fiber and outside; but even gross deviations from equal activity coefficient in the two phases cannot alter the main conclusion that the out diffusion does not contribute significantly to the total Na outflux ( $c_{\text{Na}(i)}$  is much smaller than  $c_{\text{Na}(o)}$  and  $c_{\text{K}(o)}$  is much smaller than  $c_{\text{K}(i)}$ ;  $M_{\text{Na}(out)}$  is therefore much smaller than  $M_{\text{Na}(in)}$ ; the total influx and outflux, however, are the same). But it still remains to be decided what part if any is played by exchange diffusion before we know the rate of active Na transport. Krogh's hypothesis (80) that the Na outflux is identical with the transport is naturally very attractive; but in the case of muscle, Conway's calculations (15) indicate that the Na extrusion must be slower than indicated by the exchange of radio sodium. The striated muscle presents unusually good material for deciding whether it is energetically possible to identify the Na flux with the transport, because the resting metabolism is low and the Na exchange high. This problem was the subject of the preliminary study by Levi and Ussing (83). The rate of Na exchange in the fibers was determined as follows: The isolated muscles (frogs' sartorii) were equilibrated with Ringer, containing  $\text{Na}^{24}$  for about two hours. The process of washing out the  $\text{Na}^{24}$  was then followed over a period of several hours. The logarithms of the counts of  $\text{Na}^{24}$ , washed out in equally long periods, when plotted against time, lie on a curve which can be resolved in two straight lines. The slopes of these lines are taken to indicate the rates of Na exchange between washing fluid and interspaces, and between interspaces and fibers, respectively. If this interpretation of the result is correct, the half renewal time of interspace Na is about two minutes whereas the half-renewal time for the fiber Na is about 30 minutes. Under the assumption that only the slowly exchanging Na is intracellular, the amount of Na leaving the fiber per hour can be calculated. This flux combined with the Na concentration and the resting potential were used for a calculation of the minimum energy required to expel the Na as fast as it enters, assuming no exchange diffusion. This admittedly rather rough estimate gives about 50 Cal/hr/kg. of muscle. According to Conway (15) the resting metabolism of winter frogs' sartorii is about 175 Cal/hr/kg. This means that more than 30 per cent of the energy output of the muscle would be consumed by the active Na extrusion even with a 100 per cent efficiency of the energy transfer from metabolic processes to active transport.

It will thus be seen, that although the Na outflux from the fibers may still be considered identical with the transport, it is rather unlikely that it is so. The possibility of at least some exchange diffusion has evidently to be considered. Thus we are in the situation that we may have already in the isotopes the means for the determination of true transport rates, while on the other hand we may be far from that goal if the exchange diffusion turns out to be a fast process compared with the transport.

Organs which are able to perform a net transport of Na ions (as a rule together with Cl) are widely distributed in the animal kingdom. Well-known examples are the salt reabsorption in the kidney tubules and in the intestine (60, 29, 30). Other examples are the salt secretion in the gills of the eel (68) and the salt uptake from the surrounding water by a wide variety of fresh water animals (79). In most cases the transport seems to go in one direction only. The eel, for instance, which can excrete salt when

in salt water, cannot take up salt from dilute solutions (79). There are, however, exceptions from the rule of one-sided transport. The stickleback (*Gasterosteus aculeatus*) which was found able to take up salt from fresh water (77) excretes salt when kept in sea water (42). According to Panikkar (105, 106) the brackish water prawn *Palaemonetes varians* which can live in nearly fresh water as well as in sea water is isotonic in water of about 2 per cent NaCl. This species is practically homoiosmotic, the difference in its osmotic pressure over a range of 5 per cent NaCl in the external medium being only 0.8 to 1 per cent. As the urine of this species is nearly isotonic with the blood irrespective of the nature of the medium, Panikkar concludes that the animal must take up salt in dilute medium whereas it excretes salt (and possibly takes up water) in concentrated solutions. A similar ability for osmotic regulation is found in the pacific prawn *Metapenaeus monoceros* (107).

The ability to transport salt in both directions may turn out to be quite common among crustaceans. Jones (64) thus found that out of nine different species of crabs from the west coast of North America two were able to keep up their osmotic regulation as well in dilute salt water as in salt water more concentrated than the blood of the animals. It has still to be found out whether the salt excretion and the salt uptake are performed by the same cells or whether special organs are present for the two processes. In most of the cases just mentioned the P.D. across the transporting cells has not been measured during the salt transport and therefore it is not quite certain whether it is the Na ion, the Cl ion or both which are transported. There is, however, indirect evidence that a true Na transport is involved in some of the cases of salt transport listed above. The goldfish for example may take up Na not only from NaCl (NaBr) but also from dilute solutions of  $\text{NaHCO}_3$  (78). The uptake of Cl is evidently unnecessary for the Na uptake. In one experiment with a frog it even was observed that the animal took up Na for a period of time while simultaneously losing Cl to the solution. A similar independence between Na and Cl uptake has been found to occur quite commonly in the axolotl when kept in dilute salt solutions (4).

As first pointed out by Koch (71) the salt-absorbing organs in a number of arthropods have in common the ability to concentrate Ag ions from very dilute Ag salt solutions. The uptake is indicated by the blackening of the organs (anal papillae of mosquitoes, gills of daphnia and crayfish) performing the uptake, when exposed to light. If the organs are not exposed to light the Ag uptake results in the formation of a white precipitate in the cells which is not AgCl (Koch, personal information). In so far as Ag and Na are both monovalent ions which, judging from the position of the elements in the periodical system are related, one might assume that the Ag simply takes the place of Na in the transport system. Krogh (79) therefore considered it possible that the salt uptake through the anal papillae of the mosquito larvae was in fact a Na transport with the Cl ions following by electric attraction. If this theory is correct one would expect a competition between Ag ions and Na ions in the transporting organs. As a matter of fact Holm-Jensen (56) has found that specimens of *Daphnia magna* which were killed by Ag poisoning had a considerably reduced Na content; animals killed by organic poisons showed about normal Na content. Poisoning with  $\text{Cu}^{++}$  or  $\text{Hg}^{++}$  had the same effect as Ag on the Na content of the animals. The amount of heavy metal sufficient to kill the daphnia was as low as about 0.03



mg/l. The heavy metals only exerted their toxic effect when present as ions, so that complex formers like glutathione in little more than equivalent amounts would eliminate the toxicity of the metal ions. Although these experiments are in agreement with the hypothesis that the heavy metals block the groups which would otherwise bind Na, other possible explanations have to be considered. The very fact that the heavy metals block a number of the metabolic enzymes raises the question whether it is the ion carrier or the system delivering the energy which is affected.

The salt-transporting cells of vertebrates apparently differ from those of arthropods in that they never accumulate Ag. On the other hand there are indications that the salt-transport mechanisms of different organs of vertebrates are related. The fact that the adrenal-cortex hormones influence the Na reabsorption much more than the Cl reabsorption in the kidneys might suggest a specific Na transport mechanism in the kidney tubules. The disturbance in tubular function, following adrenalectomy is shown by an inadequate Na reabsorption from the glomerular filtrate even when the concentration in the plasma is low, and secondly by the failure to excrete K and P at high concentrations when these ions are abnormally concentrated in the blood plasma (43, 87). The normal conditions can be restored by the injection of cortex extracts. Hartman *et al.* (44, 145) have succeeded in separating from the cortex extract a substance which is highly potent in its ability to cause retention of Na, but having no effect on the K excretion. It occurs in sufficient amount to account for the Na-retaining power of adrenal extract. An interesting point is that this substance binds some Na which is split off as NaCl on treatment with 0.1 normal HCl together with simultaneous loss of a considerable proportion of the potency. Although this Na binding may be coincidental it might be worth while considering whether the Na factor of the adrenals is identical with or is part of the Na carrier in the Na transport system.

The salt absorption in the gut of dogs is also impaired after adrenalectomy (31). The absorption of Na as well as of K and Cl falls after the operation. It is important to note, however, that relative to the Na uptake the K uptake is increased after the operation. In several cases there was even an increase in the Na content of the gut. The experimental conditions were such that the K concentration in the gut at the beginning of the experiment was always higher than that of the blood plasma, whereas Na was taken up against a concentration gradient. It is interesting to note that even the salt uptake through the skin of amphibians seems to depend on the function of the adrenal cortex. It has been found that commercial preparations of the pressor hormone from the posterior lobe of the pituitary induces an increased uptake of NaCl through the skin of the axolotl (3). A considerable net uptake is, as a rule, observed and tracer experiments show a mean of 200 per cent increase in the Na influx. Later it turned out (75) that the adrenocorticotrophic hormone from ox pituitaries could bring about a reaction which in all respects resembled that brought about by the pressor preparation. This might indicate that corticotrophic hormone present as impurity was responsible for the pressor-fraction reaction. As already mentioned the stickleback can keep a constant salt level in the blood both in fresh water and salt water. In the mating season, however, the regulation mechanism is more or less disturbed and the animals do not stand fresh water or pure salt water very well as

stated by Koch and Heuts (74). These authors also found that the feeding of thyroxine to this fish brought about severe disturbances in the osmotic regulation if the animals were kept in sea water. The authors assume a disturbance in the normal function of the adrenal cortex to be the underlying reason for the failing osmotic regulation (73).

Only in the case of frog skin do we have definite evidence that a specific Na transport is at work. This organ will therefore be treated at some length.

The presence of a Na transporting mechanism in the isolated frog skin was strongly indicated by Huf's (58) finding that the frog skin will transport salt from the outside to the inside when bathed with Ringer on both sides. Although he did not make Na analyses, Huf evidently considered the Cl transport to be a measure of a salt transport. He speaks of "einer Kraft, die Ionen von aussen—innen bewegt". The potential measurements showed that the inside solution was always positive relative to the outside; Huf did not mention the possibility, however, that the Cl transfer might be due to electrostatic forces. Katzin (67) using the radioactive isotopes  $\text{Na}^{24}$  and  $\text{K}^{42}$  was able to demonstrate that Na passes with greater rate inward than outward through the frog skin when the same mixture of NaCl and KCl was applied on both sides. The rate inward was 60 per cent higher than the rate outward with pure 0.12 N NaCl on both sides and no less than 300 per cent greater if 80 per cent of the NaCl was replaced by KCl (all the solutions were 0.12 N as to Cl). The experiments with  $\text{K}^{42}$  indicated that there was a net movement of K outward, at least at the lower range of K concentrations. The net transfer of Na inward was about  $12 \text{ to } 20 \times 10^{-8} \text{ eq/hr/sq. cm.}$ , whereas the net transfer of K outward was  $2 \text{ to } 6 \times 10^{-8} \text{ eq.}$

Now, it turns out that the inside of the skin is positive relative to the outside under all experimental conditions where a salt transport can be observed; this can only mean that at least the Na ions are transported actively, whereas the Cl ions might follow passively.

Evidence that the frog skin preparations can transport Na, not only against a potential gradient, but also against a steep concentration gradient was given by Ussing (149, 150). The skin was placed as a membrane between two salt solutions (that on the inside being always Ringer's) and the outflux or influx of Na determined with  $\text{Na}^{24}$ . Concurrently with the flux determinations, the P.D. between the solutions was measured. It was found that the influx is definitely much higher than the outflux even down to about 1 millimolar NaCl on the outside. This means that there is a net uptake of Na in the entire region studied. When the outside concentration is above 10 millimolar, the Na influx may simply be used without serious error as a measure of the net Na transport and we thus have a relatively simple and convenient way of determining the influence of different factors on the transport system.

An interesting point is that the Cl influx is smaller and as a rule much smaller than the corresponding Na influx. This means that part of the Na removed from the outside solution must be replaced by another cation. Preliminary experiments indicate that the ion in question is the H ion. To a lesser extent K (139, 67) and Ca (76) are also lost through the skin. Steinbach (139) found that the frog skin is in equilibrium (as to K) when the solution applied to the inside is 0.0025M in K whereas

the solution has to be 0.01M when applied to the outside. With the same solution on both sides of the skin there will therefore be a constant net outflux of K. This is probably an entirely passive process since the P.D. will force out the K. It is possible that even the Cl ions move passively; in this case the transport of sodium ions across the skin might be considered responsible for the P.D. If both sides of the skin are bathed with the same solution, work has to be done on the Cl ions simply to overcome the frictional forces in the membrane, and as Na is transported faster than the Cl ions can follow, the inside solution will attain a positive charge. If a passive Cl uptake has to take place against a concentration gradient the P.D. required will be that giving the Cl ion the same electrochemical potential in both media plus some excess to overcome the friction. If the outside NaCl concentration is higher than 2 to 5 millimol NaCl and lower than that of Ringer, the P.D. is as a rule found sufficiently high (50–80 mV) to account for a passive Cl transport inward. Simultaneous determinations of influx and outflux of Cl as related to the P.D. would tell whether a special Cl transport mechanism is in action or whether the electric force is solely responsible for the Cl uptake.

To a large extent the behavior of the isolated frog skin toward ions can be explained by assuming that only the Na ion is transported; some evidence is present, however, that in the living frog at least there is also some active transfer of Cl (see below).

The close relationship between Na transport and P.D. is borne out by the fact that they vary in much the same way on changes in the experimental conditions. Especially illuminating is the influence of the  $pH$  on Na transport and P.D. Whereas the  $pH$  of the outside solution can be varied for instance from 6 to 8 without any effect on Na influx or P.D. both of them show a high degree of dependency on inside  $pH$ . If the  $pH$  of the inside solution is increased, for example, from 7 to 8 the Na influx increases often as much as three times whereas the P.D. may increase up to 60 mV (the inside becoming still more positive relative to the outside). Meyer and Bernfeld (97) have pointed out that the inside of the frog skin reacts to  $pH$  changes much like a glass electrode, giving an increase near the theoretical 58 mV shift for 1  $pH$  unit. They ascribe the reaction to some layer at the inside of the epithelial cells being selectively permeable to H ions. Strictly speaking the H selective layer is also permeable to a number of other ions; but only  $H^+$  (or  $OH^-$ ) participate measurably in the electricity transport. Meyer and Bernfeld assume the P.D. to be brought about in part at least by the formation of H ions within the epithelial cells. These H ions by their diffusion through the H selective layer are assumed to give a positive charge to the inside solution. This explanation cannot be entirely correct because a P.D. brought about by inward diffusion of H ions would give a Teorell effect (147) forcing Na outward. If the H selective membrane hypothesis is to be kept (Meyer and Bernfeld's experiments seem to be conclusive) it can only mean that the Na transport consists in a forced exchange of Na against H ions across the H selective membrane at such a rate that the cellular  $pH$  is kept constant (149, 150). Then we would have exactly the same situation as in a glass electrode where the H selective membrane separates a solution of constant  $pH$  from one where the  $pH$  is varied.

It is interesting to note that the ultimate result of a forced  $H^+ - Na^+$  exchange

depends on the permeability of the membrane to the different free ions present. In case the membrane had been just as impermeable to  $H^+$  as it is to  $Na^+$ , no P.D. and no salt transport would have been brought about, but the outside NaCl solution would have been turned into a solution of hydrochloric acid.

The poisoning of frog skin with cyanide stops the active ion transport (58, 150) and parallel with the fall in transport goes a drop in P.D. This indicates the importance of the oxidative metabolism for the transport process. Huf (58) tried to identify still further the reaction responsible for the chloride transport and the P.D. He showed that whereas poisoning with monobromoacetate would stop the transport and depress the P.D., the addition of lactate or pyruvate to the monobromoacetate-poisoned skin could restore, in part at least both P.D. and chloride transport. This result seemed to indicate that the process responsible for the salt transport was connected with the pyruvate oxidation. Francis and Gatty (34), however, have shown that the P.D. of monobromoacetate-poisoned frog skin can also be restored by addition of salts of a number of other organic acids (for instance acetate, propionate and butyrate). Although these authors did not determine whether the substances mentioned were able to restore the salt transport, it is likely that the effect of these acids is similar to that of pyruvate. It thus seems that a number of hydrogen donors can supply the fuel for the transport reactions.

As mentioned above, heavy metals appeared to poison the NaCl uptake in *Daphnia* (56). Lundegårdh and Burström also found heavy metals to be extremely poisonous to the salt uptake in wheat roots (89). I have therefore made some experiments to find out whether  $Cu^{++}$ , as a representative of the heavy metals is a specific poison for the Na transport in the frog skin. The result was rather surprising. The presence of as little as  $10^{-6}$  moles/l of  $Cu^{++}$  in the Ringer bathing both sides of the skin brings about a steady increase in P.D. which after 5 hours may reach values as high as 135 mV, about double the value found for the controls. Later the P.D. declines, but even after 18 hours the skin is still alive as evidenced by a positive P.D. The Cu turned out to bring about a tightening of the membranes which decreased the shortening of the P.D. through the skin. It thus would appear that  $Cu^{++}$  rather stimulates the ion transport.

A stimulus may also originate in the nervous system. Steggerda and Ponder (138) found that the injection of very small amounts of strychnine sulfate into living frogs caused the P.D. across the skin to increase violently. In one experiment the P.D. rose from an initial 47 mV to 178 mV 12 minutes after the strychnine infection. It would be very interesting to know how this P.D. change is related to the movements of ions across the skin. The same authors also found that damage to the central nervous system induced a fall in P.D. It is possible that this effect was caused by the release of adrenaline or sympathine from the nerve endings. Barker Jørgensen (2) has shown that adrenaline even diluted  $1/10^6$  will bring about an enormous increase in salt permeability when applied to the inside of the isolated skin. The permeability change is accompanied by characteristic changes in P.D. (149). The use of tracer Na revealed, however, that the adrenaline has still another effect, namely to increase the active Na transport. Whether this change in Na influx is primarily due to a stimulation of the cellular metabolism induced by adrenaline or

whether the increase in influx is a secondary effect of the permeability change cannot be decided at present.

The skin is only sensitive to adrenaline applied from the chorion side just as it is far more sensitive to pH changes on the chorion side. This makes it rather likely that it is the basal cell membrane of the epithelial cells which is responsible for the active Na transport. As we have seen already many cells like muscle fibers and nerve fibers must extrude Na through their surface. As far as Na transport is concerned the difference between these cell types and the epithelial cells is that the latter are only extruding Na through the cell membrane turning inward whereas the others do this through their entire surface.

The specificity of the mechanism carrying Na across the skin was clearly demonstrated already by Krogh (76) who found that frogs in need of salt took up Na but not K or Ca from solutions of their chlorides. A most remarkable thing about this transport is that the Na is transported right through epithelial cells having a high content of potassium. Rubin (124) analyzed the skin of *Rana pipiens* for inorganic ions. The K content was 132.4 mg. per cent as compared with 90.6 mg per cent of Na. As the chorion is made up mainly of connective tissue with large interspaces containing NaCl but little K, the cells, particularly the epithelial cells, are bound to have a K content which is far higher than their Na content. It is thus safe to assume that Na is transported out through the basal part of the epithelial cells rather than transported into the cells through the membrane turning outward. While the Na is transported in, the K is slowly leaking out.

It may be appropriate to devote a few remarks to the relationship between ion transport and water movement through the frog skin. Already in 1892 Reid (118) observed a very slow fluid transfer across the skin from the outside to the inside when both sides were in contact with Ringer's solution; this observation has since been confirmed by several investigators (59). By some authors this water transfer has been termed irreversible permeability. With heavy water as a tracer, however, Hevesy, Hofer and Krogh (46) were able to demonstrate that the influx and outflux of water are about equal, the net uptake of water being only a small fraction (less than 2 per cent of the total water exchange).

The net uptake of water could be stopped if an isosmotic sugar solution was used as outside medium, so that there could be little doubt that osmosis played a major rôle in the water movement. The net uptake of water was, however, about 5 times the value calculated under the assumption that the influx and the outflux of water (as measured with D<sub>2</sub>O) were proportional to the water activities in the medium and in the frog, respectively. At that time, 1935, no really satisfactory explanation of the discrepancy could be given; but later Krogh (79) pointed out that some water may be carried along with the salt which is transported through the skin. Thus there is no reason for assuming any specific water transport mechanism. It is interesting that even in the case of the aqueous humor of the eye, the water exchange is very high compared with the net transfer, so that the water transport can be regarded as the result of the electrolyte transport (69, 70). Parenthetically it should be mentioned that the assumption on which the calculations of Hevesy *et al.* were based is not absolutely correct. If the water forms a continuous phase through the mem-

brane all diffusing water molecules will have superimposed upon their diffusion rate the mean rate of the water in the membrane; molecules diffusing upstream will be retarded and molecules diffusing downstream will be accelerated. Calculations show (151) that for dilute solutions the net osmotic uptake is about twice that calculated according to Hevesy *et al.* A similar effect can be predicted for all substances diffusing in the water phase. For small ions at low concentrations the effect is insignificant, however. It is the high concentration of water that emphasizes the importance of this streaming effect. In organs like the gut where a rapid water uptake may take place the streaming effect on the solutes may become important, so that the calculated electrochemical potentials are no longer adequate expressions for the tendency of ions to move in one direction or the other.<sup>3</sup>

*Transport of Hydrogen Ions.* It will have been noticed that the transport of potassium into yeast cells might also be called an excretion of hydrogen ions. Similarly the sodium transport through the frog skin could most properly be described as a forced exchange of Na against H. Indeed, most cases of cation transport are probably connected with a compensatory transfer of  $H^+$  (in some cases perhaps of  $NH_4^+$ ). Thus, according to Pitts *et al.* (110, 111, 112, 113) a forced exchange of  $Na^+$  against  $H^+$  is probably responsible for the acidification of the tubular urine. In experiments on dogs it was conclusively shown that, under appropriate conditions, the quantity of acid excreted was much larger than that which had filtered through the glomeruli. This means that either an acid is excreted and its anion reabsorbed together with  $Na^+$  or there is simply an exchange of  $Na^+$  against  $H^+$ . Actually these two possibilities can hardly be distinguished from each other. A  $Na^+-H^+$  exchange would probably require an anionic carrier pendulating back and forth across the cell membrane of the tubule cells, so that, in a way, an acid goes to the tubular urine and a Na salt goes back (134, 125).

The acidification of the urine probably takes place in the distal tubuli as distinguished from the main absorption of NaCl which is performed in the proximal tubuli. At least in the frog (99) the acidification takes place in a short segment near the distal end of the distal convolution. In frogs no acidification is observed if the carbonic anhydrase of the tubuli is poisoned with sulphonamide (53). Höber therefore assumed the acidification to be due to reabsorption of bicarbonate ions. In the experiments with dogs, however, Pitts *et al.* (111) found that the acid elimination continued although at a slower rate when the carbonic anhydrase was inhibited by sulphonamide. Therefore, according to Davenport (24), it is likely that the carbonic anhydrase has only the function to place the necessary amount of  $H^+$  at the disposal of the  $Na^+-H^+$  exchange system by forming  $H_2CO_3$  from  $CO_2$ . Davenport suggests that a forced  $Na^+-H^+$  exchange is also responsible for the formation of the hydrochloric acid of the gastric juice. The ions originate mainly from the  $CO_2$  formed in the parietal cells, but at least in warm-blooded animals the carbonic anhydrase is of little importance for the process because the spontaneous hydration of  $CO_2$  suffices to form the necessary amount of H ions. The main work done by the cells is, however, the excretion of the H ions.

<sup>3</sup> In the tracer experiments of Visscher *et al.* (153) there was no simple relationship between the osmotic pressure in the gut and the movements of ions and water.

A very similar hypothesis has been advanced by Conway *et al.* (17, 18). They also consider the essential process in the formation of the HCl to be exchange of  $H^+$  for inorganic cations. A solution of inorganic chloride is supposed to flow past an exchange region and the reabsorbed inorganic cations are returned to the blood stream or to the alkali chloride-secreting part of the cell. By analogy with the ion exchange in yeast these authors assumed the chloride to be KCl, but in a later paper (16) Conway points out that it might be NaCl since a Na transport is known in many other animal cells. The hypothesis of a  $Na^+H^+$  exchange being the crucial step in the formation of the acid is in good accord with the known facts about the acid formation. According to Davenport (24) the secretion is in osmotic equilibrium with the blood so that no water transport need be postulated. The fact that Br and, to some extent, I can replace Cl without any effect on the secretion rate speaks in favor of a passive transfer of the chloride ions (25).

Across the wall of the resting stomach there is a P.D. which in frogs is about 50 mV (98, 22) and in dogs 70–95 mV (116). When the secretion of gastric juice sets in (for instance on histamine stimulation) the P.D. drops considerably.<sup>4</sup> In case the stomach wall had been permeable to  $H^+$  but impermeable to  $Na^+$  the P.D. would have been determined by the proportion between the  $H^+$  concentration in the gastric juice and in the blood;  $58 \cdot \log \frac{10^{-1.4}}{10^{-7.4}} = 350$  mV. We must therefore assume either that the stomach wall is impermeable to  $H^+$  or that  $Na^+$  diffuses about as fast as does the H ion so that the diffusion potentials nearly cancel out. Tracer experiments with Na have shown that the stomach wall is permeable to  $Na^+$  although the permeability in the acid-excreting part is very low compared with the permeability in other parts of the stomach (21). The electrochemical potential of the H ion is evidently very much higher in the gastric juice than in the blood so that an active  $H^+$  transport must take place. The fact that the mucosa side is some 20 mV negative relative to the serosa side means that the electrochemical potential of the Cl ion is also somewhat higher in the juice than in the blood, although the concentration of Cl is about the same in serum and gastric juice. This would indicate that an active Cl transport is also involved in the secretory process. However, the P.D. which we can measure is the resultant of the P.D. values across all the cells of the gastric mucosa. If, as there is good reason to believe (86), the acid is only elaborated by the parietal cells, the P.D. across the canaliculi may differ considerably from the 'gross P.D.' which is the only value available to us. The reasonably safe conclusion from the P.D. measurements is that a  $H^+$  transport has to take place whereas the  $Cl^-$  may or may not be transported.

It should be pointed out, however, that the  $Na^+H^+$  exchange hypothesis is far from being generally accepted. Despite the enormous amount of work done in the field our knowledge is still so incomplete that a number of widely different hypotheses seem to fit the known facts equally well. Hollander (55) whose article gives a thorough discussion of the relevant literature up to 1943 mentions as a possibility that the hydrochloric acid is formed by membrane hydrolysis of alkali chloride. A contractile mechanism, wholly or in part identical with the pericellular membrane is as-

<sup>4</sup> For gastric potentials in human beings see (120).

sumed to exert an intermittent pressure on the cell contents. If the membrane of the canaliculi is taken to be cation impermeable, the  $\text{Cl}^-$  ion can only pass out if a corresponding number of  $\text{OH}^-$  ions pass in so that, in the end,  $\text{HCl}$  is formed in the secretion and alkali chloride remains in the cell.

Bull and Gray (12) proposed the interesting hypothesis that a nearly pure solution of some organic acid, such as pyruvic acid, is secreted into the basal end of the canaliculi. As the secretion passes through the canaliculus the anion diffuses back into the cell where the metabolism keeps its concentration at an extremely low level. The  $\text{H}^+$  ions cannot follow, however, because the membrane is theoretically cation impermeable. To maintain electroneutrality,  $\text{Cl}^-$  has therefore to replace the organic anions that diffuse away from the secretion.

Other hypotheses where the  $\text{HCl}$  formation is thought to be brought about by an electric current originating from some metabolic process, have been advanced by Rehm (116, 117) and Davis, Longmuir and Crane (22, 26).

A discussion of the merits and drawbacks of these different hypotheses is hardly warranted because too many unknowns enter into the premises. More work is evidently needed before we can hope to understand the apparently simple formation of  $\text{HCl}$  from  $\text{NaCl}$ . The use of new approaches can still bring astonishing results. Thus it may be mentioned that Teorell *et al.* (85) have found the primary acidity of the secretion to vary between 170 mN and 350 mN whereas hitherto it has been generally accepted that the secretion was isotonic with the blood and of constant acidity.

#### ACTIVE TRANSPORT OF ANIONS

*Transport of Anions in the Animal Organism.* The transport of phosphate in animal cells has been referred to already in the introduction. In this article we shall not enter into a discussion of the transport of organic substances but it should be mentioned that we have examples of manifest anion transport in the excretion of a substance such as diodrast and certain acidic dyes like phenol red in the kidney tubules (54).

A rigorous proof that  $\text{Cl}^-$  ions are transported actively in the animal organism is still lacking. We have seen already that the low  $\text{Cl}^-$  concentration in muscle and nerve can be explained as the result of a Donnan equilibrium. The uptake of  $\text{Cl}^-$  in many fresh water organisms is as a rule accompanied by a  $\text{Na}^+$  uptake which we have good reason to believe is of active nature, so that a specific  $\text{Cl}^-$  transport need not be postulated. In living frogs, however, Krogh (78) found an uptake of  $\text{Cl}^-$  from  $\text{KCl}$ ,  $\text{CaCl}_2$  and  $\text{NH}_4\text{Cl}$  solutions;  $\text{K}^+$ ,  $\text{Ca}^{++}$  and  $\text{NH}_4^+$  were not taken up and no significant amount of  $\text{Na}^+$  was present. The  $\text{Cl}^-$  uptake seemed to be an exchange against  $\text{HCO}_3^-$ . These experiments certainly speak much in favor of a  $\text{Cl}^-$ - $\text{HCO}_3^-$  exchange mechanism. Quite recently Barker Jørgensen and Levi (4) have given substantial support to the idea that under certain conditions some exchange of anions is aiding in the uptake of  $\text{Cl}^-$ . They found that frogs which had stayed in a slow stream of dilute  $\text{NaHCO}_3$  for some time showed a net uptake from a  $\text{NaCl}$  solution of  $\text{Cl}^-$  as well as a total  $\text{Cl}^-$  influx which were well in excess over the corresponding values for  $\text{Na}^+$ . In their experiments with salt uptake in the gut, Ingraham and Visscher (62) observed that the  $\text{Cl}^-$  uptake was in excess over the  $\text{Na}^+$  uptake in many cases.



At the same time the bicarbonate concentration of the gut content showed an increase. A  $\text{Cl-HCO}_3$  exchange would explain this result. The authors point out, however, that the secretion of the intestinal glands contains considerable amounts of  $\text{NaHCO}_3$ . If  $\text{Na}^+$  and  $\text{Cl}^-$  were taken up in equivalent amounts this would lead to an apparent excess uptake of  $\text{Cl}^-$  in exchange for  $\text{HCO}_3^-$ . The secretion of the skin glands of frogs and fishes have not been sufficiently studied to indicate whether enough  $\text{NaHCO}_3$  is produced to account for the apparent  $\text{Cl-HCO}_3$  exchange. But for the time being the existence of an active  $\text{Cl}^-$  transport in the frog skin cannot be ruled out.

An active concentration of inorganic iodide takes place in the thyroid gland. Also the higher homologue of iodine, ekaiodine is concentrated by the gland (40) and so is to a lower extent bromine (109, 5); but it is not known whether the last two elements are present to any extent as ions or only in inorganic compounds.

Mann, Leblond and Warren (96) demonstrated that about 5 per cent of the I of the thyroid of the normal dog was inorganic iodide. This I could not have been formed from the organic iodine compounds during the isolation process. In experiments where weightless amounts of tracer iodine were given to the dogs it turned out that, at any time, the relative specific activity of the iodine of the diiodotyrosine was higher than that of the free  $\text{I}^-$ . This indicates that the major part of the I of the diiodotyrosine does not come from the free iodide of the gland. Possibly the reaction which introduces the iodine in the tyrosine molecule takes place in the surface of the thyroid cells, and at least some of the free iodide of the gland is formed by the splitting off of some of the organically bound iodine.

Later observations are difficult to reconcile with this hypothesis. Thus Leblond (82) found that when large doses of iodide are introduced in a guinea pig the larger part of the iodine fixed by the gland remains in the form of inorganic iodide for some time. Franklin and Chaikoff (36) have found that the formation of radioiodotyrosine and radiothyroxine in thyroid slices is inhibited by sulfanilamide and related compounds in  $10^{-3}$  M solution. At this concentration of sulphonamides the capacity of the surviving thyroid slices to take up iodine from the solution was not depressed. It thus appears that for uptake of I there is another route than that over diiodotyrosine. The thyroids of rats made goiterous by prolonged feeding of propylthiouracil possess the I concentrating mechanism despite a complete block of the formation of organic iodine compounds (146). In fact the capacity of goiterous glands for fixing inorganic iodide is much greater than normal. A linear relation was found between the iodide concentration in the thyroid and plasma, the concentration factor being 200 to 300. The uptake of iodide by the goiterous gland is inhibited by KCNS (146, 152); but the CNS ion is but little concentrated if at all. Although the iodide might exist in the gland in a very loosely bound form (128), it is just as likely that an active transfer of iodide takes place and that it is the transport mechanism which is blocked by CNS. The presence of the I in free rather than in bound form would explain that the iodide is freely dialysable and ultrafiltrable and that it is not coprecipitated with the protein of the gland when the tissue is homogenized with protein precipitants (146).

*Transport of Anions in Plants.* 1) *The ion uptake in plant roots.* Excised roots of young plants of the grass family possess a pronounced ability to take up ions even

from dilute solutions. This process has been studied very carefully by different groups of investigators, but so far no unanimously accepted view has been obtained as to the mechanism involved. Lundegårdh, as well known (90, 92, 94) assumes that the process is essentially an anion uptake whereas the cations are supposed to be electrically attracted. Hoagland and Steward (49, 50), on the other hand, do not consider Lundegårdh's theory convincing and they apparently prefer the assumption of a cation transport as well as an anion transport. As a matter of fact the ion uptake observed is the resultant of two different phenomena, namely an ion accumulation in the cell sap of the roots' parenchyma and a transport of ions from the outside solution to the wood vessels, where they contribute to the formation of the bleeding sap. Although we do not know whether both of these phenomena are brought about by the same mechanism, there seems to be a rather close correlation between them. Hoagland and Broyer (48) have shown that the concentrations of K, Na and Br in the cell sap and bleeding sap from barley roots vary in very much the same way with time, when the roots are transferred from distilled water to an experimental solution containing these ions. The ion concentrations are, however, always higher in the cell sap than in the bleeding sap. Naturally a formal similarity in the rates of ion uptake in cell sap and bleeding sap is not sufficient to prove that both processes are due to a common mechanism. Especially Arisz (1) has stressed the importance of treating the transfer across the cell membrane and across the tonoplast separately. The potential measurements which have been performed by Lundegårdh (91, 92, 93) and others (130) only relate to the P.D. between cell sap and bleeding sap. We are without any direct knowledge as to the P.D. between cell sap and protoplasm as well as to that between the epidermal cells and the medium. Lundegårdh has not stressed this point sufficiently. In fact he assumes that the whole potential difference is located at the boundary between the epidermal cells and the medium (93). In view of the fact that a number of cell membranes, each the possible seat of a P.D., are interposed between the outer membrane of the epidermal cells and the bleeding sap, this assumption is not very well founded. Lundegårdh himself (94) believes that there is an anatomical-physiological differentiation of the root parenchyma so that the power of ion uptake declines from the outside inward. Such a differentiation most likely would involve potential differences between the cells. For the time being the estimation of these potentials is hardly technically possible. Nevertheless Lundegårdh's potentiometric studies are of great value for our present inquiry as to which ions are actively transported; but we have to confine this discussion to the transport from medium to bleeding sap. The latter (94) is a dilute salt solution practically free of organic matter. The dominating ions are  $K^+$  and  $NO_3^-$  which also are the ions most readily taken up from the medium; but almost any ion which is not too poisonous is taken up. As already shown by Laine (81) the concentration of any cation in the bleeding sap depends on the concentration of that ion in the medium in a way that recalls Freundlich's adsorption isotherm. The salt concentration in the bleeding sap is higher than that of the medium at low outside concentrations, but as the outside concentration is increased the relative increase in the bleeding sap becomes less and less. We may take as an example one of Lundegårdh's experiments (94). It turned out that below about 30 millimoles  $KNO_3$  in the medium the bleeding sap is more concentrated as to  $K^+$  and  $NO_3^-$  than the medium. With a one milli-

molar solution the  $\text{KNO}_3$  has been concentrated about 13 times. It is quite evident that work has been done on the  $\text{NO}_3$  ion. Not only has it been concentrated, but the concentration has taken place against a P.D. which may be estimated to have been some 52 mV (91). This P.D. on the other hand would suffice to account for at least the better part of the  $\text{K}^+$  concentration. About 65 mV would give nearly equal electrochemical potential in medium and bleeding sap. In view of the fact that the data are taken from different experiments the accordance between theory and experiment should not be stressed too much; but it seems not unreasonable that the K transport is due to electrical forces. The P.D. of the roots is primarily determined by the pH of the outside solution (91, 92). This suggests a permeability to free H ions which is much higher than the permeability to other ions. Moreover the P.D. depends on the outside salt concentration, decreasing from 150 to 200 mV in distilled water to about 60 mV in a millimolar solution of KCl or  $\text{KNO}_3$ . Whatever the origin of the P.D. it is obvious that it suffices or at least contributes substantially to bring about the cation uptake even from very dilute solutions.

In the cytoplasm and the cell sap, however, the  $\text{K}^+$  concentration is much higher than in the bleeding sap. It is clear that if the  $\text{K}^+$  concentration is brought about by electric attraction, the P.D. between medium and cell sap must be considerably higher than that between medium and bleeding sap. Considering the fact that certain plant cells like yeast and *Valonia* are known to concentrate  $\text{K}^+$  actively it is hardly justified to take definite standpoint as to whether the high  $\text{K}^+$  concentration in the cell sap is also due to anion transport or whether a specific  $\text{K}^+$  transport is responsible.

Shuffelen and Loosjes (130) have derived a formula expressing the cation uptake in roots as a function of the concentration and the P.D., with a correction for the ions that are adsorbed to the root surface. The formula seems to be in good accord with the experimental data. As, however, the uptakes refer to the total root and the P.D. is measured according to Lundegårdh and Burström between the cut end of the root (bleeding sap) and the medium, the verification of the formula must either be incidental, or, more likely the P.D. between bleeding sap and medium must be closely correlated to the P.D. between cell sap and medium.

The space does not allow a detailed discussion of the vast number of papers concerned with salt uptake in plant roots. The reader is referred to the excellent general treatments of the subject given by Hoagland (47), Arisz (1), Lundegårdh (94) and Steward (141). Only some points which may have a bearing on the transport mechanism will be presented.

The ion uptake is generally considered as a strictly aerobic process, although a small and irregular anaerobic ion uptake has been reported (94). Cyanide in low concentrations prevents salt accumulation in barley roots (48) and in wheat roots (89). An interesting property of the transport system is that methylene blue destroys the power of salt accumulation without decrease in  $\text{CO}_2$  production (48). These facts are taken by Hoagland and Broyer (48) to indicate that the salt accumulation is linked with a metal catalyzed aerobic respiration system. A similar viewpoint was also advanced by Lundegårdh and Burström (89). Lack of oxygen stops the uptake of salts as well as of water in barley roots (48). This does not prove definitely, however, that the transport mechanism can only work in the presence of

molecular oxygen. By favoring fermentation low oxygen pressure brings about an increased formation of organic acids with a resulting decrease in  $pH$ , and low  $pH$  is known to lower the permeability of many cell membranes (7, 108, 102). A number of observations show that a high  $CO_2$  tension (causing a fall in cellular  $pH$ ) will also depress the uptake of salts and water in roots (13, 48).

The ion transport in plant roots shows a low degree of specificity. A large number of different anions and cations are taken up. If the cation uptake is due to electric attraction it is no wonder that the uptake is mainly determined by the size and charge of the ions. But even the anion transport shows hardly any specificity although for instance  $NO_3^-$  is taken up by wheat roots about 4 times faster than  $SO_4^{--}$  (90).

The metabolism of the roots shows a pronounced dependence on the rate of salt uptake. As well known Lundegårdh and Burström (89, 90) assume the increase in metabolism to be determined mainly by the amount of anions taken up. This view has not been generally adopted, however. It seems certain that by a process as yet unknown, the amount of cations taken up (especially K) determines the rate of formation of organic acids (mainly malic acid) in the cells. This acid formation is so regulated that the cellular  $pH$  is maintained fairly constant (47).

2) *Salt Uptake in Halicystis*. Using a very elegant perfusion technique Blinks (7, 8) has been able to make electrometrical studies of the protoplasm-sap interphase as well as of the outside surface of this marine alga. Exact chemical analyses of the protoplasm are not available, but it is assumed that the  $Cl$  ions are to a large extent replaced by organic acids. The P.D. between the sea water and the protoplasm may thus be regarded mainly as a Donnan potential,  $Cl^-$  being the only penetrating anion. In the sap the organic acids are virtually absent. Blinks assumed, however, that "there is an active accumulatory mechanism which is constantly aiding the depletion of chloride from the protoplasm which is only a way station towards the vacuolar depot." Although the  $Cl^-$  concentration is only little higher in the sap than in the sea water the maintenance and increase of this  $Cl^-$  concentration requires work because there is a constant P.D. of about 70 mV to be overcome. Ultimately this  $Cl^-$  transport should probably be held responsible for the P.D. between the outside and the lumen when both membranes are in contact with sea water.

Whether or not there is also a cation transport cannot be said with certainty. The P.D. in *H. ovalis* seems to be sufficiently high to account for the high  $K^+$  concentration in the sap of this species, and *H. Osterhoutii* does not concentrate  $K^+$  at all. A number of factors influence the P.D. and presumably the ion transport in *Halicystis*. The P.D. falls to very low values when the organism is subjected to low  $O_2$  tension. At the same time the electric resistance of the membranes increases violently. A similar reaction is brought about by weak acids like  $CO_2$  and acetic acid and by prolonged stay at low temperature. In all cases, according to Blinks (7, 8) the explanation seems to be a fall in membrane permeability, brought about by a more acid reaction of the protoplasm. This behavior of the cell membranes makes it very difficult to decide whether the ion transporting mechanism is in itself strictly aerobic or whether the fall in transport at low  $O_2$  tension is simply due to permeability changes.

3) *Ion Uptake in the Characeae*. As already mentioned (104) the cell sap of the

fresh water characean *Nitella* is normally negative relative to the outside solution. When placed in a solution containing 9 millimoles NaCl and one millimole KCl/l. the P.D. was estimated to be about 100 mV. The high concentration of cations, notably  $K^+$  in the sap may therefore be the result of electric attraction, whereas the anions of which the chloride ions make up the dominating fraction have to be transported actively. *Nitella* can live and still take up salts from solutions which are much more dilute as to salts than that mentioned above. It cannot be decided for the time being whether the P.D. of the cells in these very dilute solutions will still suffice to bring about the cation uptake. In their study of the permeability of the membranes of *Nitella* and *Tolypellopsis* Holm-Jensen, Krogh and Wartiovaara (57) consider the exchanges of  $K^+$  and  $Na^+$  ions to be due to an active transport into the protoplasm and a passive diffusion out. Although this interpretation of the results may be correct the possibility of passive diffusion between protoplasm and surroundings cannot be ruled out. The interesting attempt made in this paper (57) to calculate the relative permeabilities of the outer and inner plasma membranes are based on the assumption that the diffusion rates are solely determined by the concentration gradients of the ions; whether or not the P.D. suffices to bring about the cation uptake, the potentials will certainly modify the diffusion rates. If, as Osterhout (104) has found, the main part of the P.D. is between plasma and cell sap it might be suggested that at least part of the anion transport takes place across this structure. Hoagland and Broyer (48) in experiments with radio-Br found this ion to approach a higher activity in the cell sap than in the plasma; this they take to indicate a secretory process transferring Br (and Cl) from the protoplasm to the sap.

#### CONCLUSION

It has been one of the main objectives of the present paper to point out that specific chemical reactions between certain inorganic ions and organic cell constituents is of decisive importance for the ion transport processes. Although the pore size of the membranes and electric potentials resulting from metabolic processes are bound to play a major part in determining the ultimate result of the specific transport processes, pores and potentials cannot alone explain the ion distributions found.

The problem of specific ion transport should conceivably be attacked along similar lines to those used to characterize the metabolic enzymes, so that we must find specific poisons for the different steps involved. But for the time being the major problem remains to determine which ions are actively and specifically transported. The study of the cellular metabolism has taught us a lesson which is well worth remembering even in the study of active transport. What seemed to be a simple combustion of organic matter turned out to be the resultant of an enormous number of enzyme reactions. Similarly, the number of ion transport mechanisms may also be very large; but in certain organs some process or other may be vastly superior to the others in determining the ion movements and in such organs the particular systems may be studied.

It is the belief of the author that the combined use of tracers and electrophysiological techniques will prove very useful to determine which specific transport processes are predominant in different cells and tissues.

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# DEVELOPMENT OF ACUTE TISSUE DAMAGE DUE TO COLD

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PEOPLE INHABITING AREAS with cold climates learn empirically to protect themselves against the injurious effects of low temperatures. In these areas acute damage due to cold is usually limited to victims of accidents, cases of acute alcoholism, and to inexperienced city dwellers hiking in the mountains during winter. In war, however, the picture changes. Under such conditions people may for shorter or longer periods be placed in circumstances where even the experienced are unable to take precautions; in addition, large groups of men who are unfamiliar with life at low temperatures may be exposed to cold for long periods. It is, therefore, a peculiar fact that the problems of tissue damage due to severe cold have not primarily occupied students in the northern countries, but mainly those of the larger warring nations, and that the clinical and experimental studies belong to periods of the great wars.

That exposure to cold may produce immediate and direct damage to the tissues and be followed by violent vascular reactions is an observation as old as the history of man in cold climates. Understanding of the mechanism of the different processes is, however, still limited. At the outbreak of World War II the existing state of knowledge of the subject could be summarized as follows:

- 1) Severe local damage with necrosis due to cold can occur as a result of tissues freezing to ice, as well as after prolonged exposure to low temperatures above freezing.
- 2) There seems to be a certain critical temperature below which the tissue destruction is direct and immediate.
- 3) After thawing of frozen tissues, and after the return to normal surrounding temperature of tissues subjected to prolonged cooling, violent vascular reactions occur.
- 4) Inside the vessels corpuscular aggregates have been observed, and are believed to be of importance for the development of necrosis, by blocking the vessels.

In the present review an attempt will be made to analyze the reactions to cold and to distinguish between damage which is immediately lethal to the cells, and damage caused by subsequent events in the tissues. Late and secondary complications like secondary thrombosis, infection, demarcation and repair will not be considered.

## PRELIMINARY AND TRANSIENT RESPONSES TO LOWERED TEMPERATURE

This problem has been extensively considered in Haxthausen's monograph (11), in Lewis's paper (27) and Stray's thesis (40). It seems to be uniformly accepted that the first reaction to a lowered external temperature is a sensation of cold, a constriction of the blood vessels of all calibers, and a contraction of the smooth muscles of the skin, causing goose-flesh. The skin is pale. If the surface temperature of the

skin falls to 25°C. the reaction changes in character, inasmuch as the minute vessels open up and are filled with blood, the larger vessels remaining contracted. The skin color is cyanotic because the tissue metabolism still is sufficiently rapid to cause a greater demand for oxygen than the slow circulation can satisfy. If the skin temperature sinks below 15°C. the skin color changes to bright pink. The hyperemia is 'arterial', partly because the tissue metabolism is brought nearly to a standstill and partly because of the incomplete dissociation of oxyhemoglobin at this low temperature. The tissue is in a state of partial hibernation. Stray describes how the goose-flesh disappears at a temperature below 7 to 8°C., and Kriege (19) found a spastic reaction of the capillaries immediately before the tissue freezes to ice.

Lewis points out that the vasoconstriction is a complex response; indeed, it comprises three separate reactions. First, there is a direct and persistent response of the superficial vessels to cold; they contract locally. Secondly, cold when applied to any part of the skin causes an immediate general vasoconstriction by reflex action through the central nervous system. This reflex, however, is only transient. It is succeeded and replaced by the third response. Cold venous blood returning from the cooled skin joins the blood in the general circulation and lowers its temperature. This blood travels to, and acts upon, a central nervous mechanism that is sensitive to cold and responds by calling forth a general and persistent vasoconstriction. The second reaction is easily displayed, for if a limb is cooled while the circulation to it is cut off there is an immediate and transient general vasoconstriction; but the third reaction takes place only when the circulation to the cooled limb is restored (32). In these vasoconstrictions, whether they are brought about locally or generally, all surface vessels—arteries, arterioles, capillaries, venules and veins—participate. The dilatation of the minute vessels, on the other hand, seems to be caused by strictly local factors.

#### PROLONGED EXPOSURE TO LOW TEMPERATURE ABOVE FREEZING

A number of workers have described the development of edema after a certain period of exposure to cold, and this is actually an everyday observation in countries with white winters. The skin becomes thickened and stiff. Lewis (28) carried out a series of experiments and concluded that "When an extremity is cooled, as by immersion in cold water (5°C.), it swells. This increase in volume occurs in both skin and subcutaneous tissue, and may amount within 3 hours to as much as 15 per cent of the original volume. The swelling is due mainly to an edema of the tissues, judged to be inflammatory from its relatively rapid outpouring and from its relatively high protein content. The contribution in the form of imbibed water is very slight. From this and correlated evidence, it seems that cold directly injures the skin and subcutaneous tissues. This effect begins at about 15° to 18°C. and increases as the scale of temperature is descended." Brown, Wise and Wheeler (3) confirmed these observations, using a more refined technique, the pressure plethysmograph. They also confirmed an older observation by Landis and Gibbon (22), that cold reduces the rate of reabsorption of previously filtered fluid. They found that this disturbance of fluid removal is associated with a reduction of the effective colloid osmotic pressure of the plasma amounting to approximately 10 cm. of water. Brown and

Landis (2) reported experiments on the effect of local cooling upon fluid movement and capillary permeability in the frog's mesentery. They concluded: "During local cooling of the frog's mesentery, no signs of capillary injury or of leakage of protein from the capillaries appeared until after the tissue had been frozen." In the experimental studies of Smith, Ritchie and Dawson (39) a marked edema was observed in rabbit extremities exposed to moderate cold, and similar observations have been made by Lange, Weiner and Boyd (24), and by the writer of this review.

There is evidently a gradual passage from the transient, physiological responses to a lowered temperature, to the reactions caused by actual damage to the tissues. It is also evident that these responses are not, in detail, the same in different animal species, but that they vary according to the degree of adaptation to the environmental circumstances.

Since many details were still obscure regarding the early reactions to low temperatures above freezing, a series of experiments was performed during the year 1948, and the results are described in a paper by Kreyberg (16) now in press. Limbs of rabbits were immersed in ice water, maintained at a temperature of approximately 2° to 5°C. Repeated measurements showed that the skin very early in the experiment reached a surface temperature of about 10°C., a temperature which persisted without variation for 24 hours. A graphic record in Lange, Weiner and Boyd's paper (24) gave a temperature of 13°C. in the deeper tissues of the limb in rabbits subjected to a similar treatment. This even temperature, considerably higher than that of the surroundings, indicates that there must be a constant blood flow, presumably through direct arterio-venous channels. It may be significant that the temperature measured in the tissues belongs to the range of temperatures which Lake (21), in his experiments, found gave the shortest survival time for tissue cultures.

The writer of this review found, moreover, that at the moment of immersion of the limb into the ice water a spasm occurs in the terminal arterioles and venules, and that concomitantly there is a dilatation of the capillary loops, close to the epidermis; hereby a sea of stagnant blood is enclosed in the surface vessels. By combining, in different ways, subcutaneous and intravenous injections of lithium carmine and fluorescein, it was demonstrated that, during the first hours of immersion in ice water, hardly a drop of blood enters this stagnant sea. After still another couple of hours, the arteriolar spasm seems to subside a little, and a trickle of blood enters; this can be recognized by a slow and faint staining of the cooled area after intravenous injection of fluorescein. After 12 hours of cooling, the entrance of fresh blood is a little freer, but still the circulation is very poor. Even after 24 hours of immersion the cooled area will take some 10 minutes to be stained, whereas the normal limb stains instantly. After subcutaneous injection of a stain in the exposed area, the degree and the speed of resorption of fluid can be followed. During the first hours the resorption is negligible, and even after 24 hours of exposure, the resorption is very slight. The hindrance to fluid drainage seems to be greater and of longer duration than the hindrance to fluid entry into the cooled area. This difference may be the cause of the initial edema which appears, and can be seen after some 4 to 5 hours of cooling. This edematous fluid is not at all stained by lithium carmine after intravenous injection of the stain, nor is the tissue stained in this early period of exposure.

After some 12 hours, however, the edema increases rapidly, and the edematous fluid, as well as the tissue, takes a strong staining by lithium carmine, in spite of the fact that the circulation is very much reduced as compared to that of the control limb. This fact, as well as the demonstration of a high protein content of the tissue fluid (1.44%), points to a new factor in the vascular response, the occurrence of an increased permeability. The edema, which initially mainly represented an accumulation of fluid because of a dynamic imbalance of outpouring and drainage of tissue fluid, is now changed to an inflammatory edema, mainly caused by an increased permeability, this again resulting from tissue damage. It may well be that, parallel with the development of increased permeability, the flow in some of the smaller vessels may also have undergone small changes, but this will be of minor importance as compared with the first factor mentioned. If the immersion is continued, the inflammatory reaction is to a great extent checked by the continued constriction of the arteries. When, however, the limb is returned to a normal surrounding temperature, this check is lifted and the full inflammatory reaction sets in.

If the reactions of the blood vessels to prolonged cooling are examined in man, in the rabbit and in the frog, one will note important differences. The cold-blooded frog, adapted to stand low temperatures as a part of its normal life, will show small changes and slight reactions. Man, the tropical animal, will show violent reactions to the exposure to cold, and the rabbit, fur-coated and used to cold and wet ground, shows a reaction of intermediate type. It may be that this more prolonged and less violent reaction of the rabbit may expose phases of a general reaction, also present in man, but obscured by the more rapid progress of the events in the latter.

The experiments of Lange, Weiner and Boyd (24) and of the present writer show that even after prolonged cooling the tissue temperature is considerably higher than that of the surrounding water, a fact not always taken into account in the interpretation of similar studies. Lake (21) found that tissue cultures kept at different temperatures showed a minimum survival time at approximately 15°C., and he explained this by the assumption that the anabolic processes at this temperature were reduced to a greater degree than the catabolic processes. At higher temperatures the two processes ran nearly parallel, while at lower temperatures both processes were brought to a standstill. The culture and the tissue came into a state of local hibernation (suspended animation). A degree of cooling involving reduction of the tissue temperature to 12 to 15°C. should accordingly bring the tissue into the range of most injurious temperature.

The occurrence of a strong inflammatory edema confirms the assumption of serious damage at this temperature range, and histological examination of the tissues shows definite evidence of degeneration. In tissue fixed immediately after termination of the exposure, Smith, Ritchie and Dawson (39) found a light vacuolisation of the epidermal cells in rabbits exposed to prolonged cooling. Blackwood and Russell (1a), in experiments with rats, found slight changes after 24 hours of exposure, but in animals examined after 48 to 96 hours of cooling, they observed polychrome staining of the fibers of striated muscles, loss of striation and an appearance of hyaline 'clods' in the fibers, reminding them of Zenker's degeneration. After prolonged exposure they also observed changes in the peripheral nerves, consisting of

irregular contours of the myelin sheaths, swelling of the cells of Schwann, and occasionally breaks in the continuity of the fibers. The present writer could not find any changes in the epidermis of rabbits after 24 hours' exposure to ice water, but the characteristic degeneration of the striated muscles was marked. The functional symptoms of damage are manifested by a tingling sensation, numbness, pains, muscular weakness and ataxy, observed in man.

When the limb is returned to the usual room temperature after prolonged cooling in ice water, the temperature of the rabbit's skin rises rapidly, at first by approximately 1°C. per minute, later more slowly. After varying intervals, dependent upon the severity of the previous exposure, the skin temperature not only reaches normal, but exceeds this. Vital staining shows an increased coloring of the exposed limb, and the picture of a more or less severe inflammation develops. In man this vascular reaction may be followed by necrosis. Again it should be stressed that not one of the usual experimental animals will react with the same severity as man, and that the reproduction of true and complete trench foot has not been accomplished experimentally: it will probably necessitate the use of more tropical animals for the tests. The author is personally convinced, however, that an efficient experiment would show that the necrosis after prolonged cooling may be two-fold in origin. First, the cooling may be so prolonged that the direct injury to the tissue is sufficient finally to kill the cells. Second, in man and animals with a similar violent vascular reaction, the damage from the cooling per se may be sufficient to cause tissue injury, but not enough to cause irreparable damage to the cells. The subsequent vascular reaction may then develop and cause a further and fatal injury to the cells.

The importance of this latter factor in man has been stressed by a number of students of these problems. Wieting (45) evidently occupying himself with trench foot, the German Nässeangrän, wrote: "Das eigentlich Wesentliche scheint uns nach allem in der schweren Schädigung der Zirkulation zu liegen, die ihrerseits in letzter Linie Folge der Kältewirkung ist. Und zwar ist es weder ein direktes Abfrieren, auch nicht eine ischämische Angrän, bei der die arterielle Kontraktion in kurzer Zeit durch Blutabsperrung den Gewebtod herbeiführt, sondern es ist ein noch protrahierteres Stadium der Kältewirkung: die Gefäßlähmung durch schwere Schädigung der Gefäßinnervation mit Schliesslicher Stase und Thrombose."

Leriche (25) touches on the same point in his statement: "Cliniquement, le gelure grave n'est pas une maladie de vasoconstriction, si vous me permettez ce paradoxe, mais une maladie de vasodilatation," and Ungley and Blackwood (43) state: "Parts which are to become gangrenous blister extensively." Kreyberg (18), in accord with Wieting, reached the following conclusion: "It is the fulminating vascular reaction which precedes the tissue necrosis; and the mechanism is probably not through pressure of an edematous fluid, not through thrombosis in its usual term, but rather through the development of stasis." This conclusion, however, still lacks experimental proof.

#### DAMAGE CAUSED BY THE FREEZING OF THE TISSUES TO ICE

Cohnheim (5), in his classical investigation of inflammation, also discusses the damage and the reactions caused by freezing of rabbits' ears to ice. He concludes

that freezing at  $-7^{\circ}$  to  $-8^{\circ}\text{C}$ . produces edema, freezing at  $-10^{\circ}$  to  $-16^{\circ}\text{C}$ . produces inflammation, and still lower temperatures, at  $-18^{\circ}$  to  $-20^{\circ}\text{C}$ ., lead to necrosis. Lake (21) finds a critical temperature at  $-6^{\circ}\text{C}$ ., a temperature which he thinks cannot be reduced without causing necrosis.

In striking contrast to these observations are the claims that animal tissues may survive temperatures down to  $-196^{\circ}\text{C}$ . even for several days. Eltorm (7) has reviewed this problem, but without strongly critical comment. The present writer does not himself feel convinced about the validity of the evidence presented for such claims. The cell reaction to vital stains is not a conclusive indicator that the cells in question are really alive. The growth of a malignant tumor after severe freezing would be conclusive, but one should demand a series of successive transplants. A single transmission may be difficult to judge. A proof would be to transplant successfully normal cells, after freezing, to a site where such cells normally do not grow. Wentscher's (44) paper of 1898 needs confirmation. Ordinary histological methods are of little help to solve this problem. The very fact that frozen sections of unfixed material may be excellent for microscopic diagnosis is based upon the remarkably small morphological disturbances produced by the freezing process. The histological changes found in clinical work on frostbite, and in similar experimental studies, are very small if the tissue is examined immediately after exposure; see Rischpler (36). Not many hours, however, may pass after exposure before marked changes may be observed.

In the older literature there seems to be a general agreement on the importance of certain vascular reactions in the development of necrosis after freezing. The mode of action has been, and evidently still is, a matter of discussion. The main German reviews of these problems between the two world wars, those of Kyrle (20) and of Ullmann (42), as well as Haxthausen's monograph (11) from Denmark, seem to center partly on the 'spastic reactions' of Marchand (30), supposed to cause an ischemic necrosis, and partly on the equally fatal results of thrombosis.

Kreyberg and Rotnes (17) in a very brief communication, put forward the observation that the blood vessels after thawing of the frozen tissue enter into stasis, with subsequent necrosis. In two further communications Rotnes and Kreyberg (37a and 37b) added details to the observations and to the technique. The main features can be followed in a very simple experiment, which necessitates only the use of carbon dioxide snow and a microscope with a magnification of 60 to 80 times. The ear of a mouse may be frozen by the carbon dioxide snow, and the happenings in the ear followed under the microscope. A drop of mineral oil on the ear makes the observation easier. Before the thawing sets in, the white crystals of ice are seen in the field. Gradually the ice melts and the tissue reappears, like a coastal landscape through fog. The blood vessels can be seen, first without any circulation, because the blood flow still is blocked by ice crystals. Gradually, however, the circulation is re-established; at first it is a little retarded, but in the course of a few minutes the blood is seen to be running quickly through vessels of all calibers. After another couple of minutes the circulation again slows down in some of the smaller veins and in the capillaries. The tissue becomes less distinct, because of the formation of edema. If a vital stain had been injected previously, the dye would appear in strong

concentration in the damaged area in this period. At the same time the red blood cells may be seen to be densely packed or in clumps and masses. After a brief interval the blood cells are found as stagnant masses in the veins and the capillaries, packed tight enough to form transparent columns, where the individuality of the single cell is lost to the eye. The circulation is brought to a complete standstill. Stasis has developed.

Brown and Landis (2) describe the development of stasis in the frog after freezing, and add: "This stasis was often reversible; the plugs of packed corpuscles were washed out by inflowing blood and normal flow was resumed." In the reviewer's experiments in mice a partial dissolution of stasis has several times been seen. One may, in the microscopic field, be able to find a vessel where the blood still is streaming, joining a vessel in stasis. Where the fresh current makes an eddy, one may observe how the erythrocytes from the area in stasis may be whirled loose and again carried into the blood stream. A limited area may then again come out of stasis: the released cells have been observed returning as individuals, and not in clumps, and we have not found any evidence of formation of any sticky film. The reason why larger areas are not similarly resolved is possibly of a hydro-dynamic nature. The area is usually too large and the caliber of the vessels too small to permit a re-establishment of the circulation. The process, as well as the term 'stasis', was familiar to the old masters of the last century: Lister (29) can be read with the greatest benefit today. In current literature, however, including most textbooks of pathology, the word 'stasis' has been given a new and less specific meaning, being synonymous with venous congestion.

Tannenberg and Fischer-Wasels (41) have given the most complete review of the problem of stasis in modern days. The theory of stasis is still a subject of discussion. Some hold that the mechanism is one of hemoconcentration only; the main causative factor is attributed to a highly increased permeability, which enables the plasma to leak out, leaving the blood cells first crowded, and then packed and stranded inside the vessels, devoid of plasma. That increased permeability is present in most, possibly in all, conditions leading to stasis is accepted by the majority of students of this phenomenon. But the increased permeability is probably not all. Lewis (26) has shown that histamine injures the capillary wall to the extent that the resulting edematous fluid has practically the composition of pure plasma. Nevertheless, stasis does not occur in connection with the histamine reaction in a normal person. Ricker (35) has been the most tireless and consistent advocate of the idea that stasis is of paramount importance for the development of necrosis after local injuries. He places changes of caliber of the different sections of the minute vessels in the foreground.

Tannenberg and Fischer-Wasels (41) regard the development of stasis as a 'colloid-chemical' problem, using the word conglutination for the clumping of the cells. They definitely discard the theory that it is a process related to blood coagulation. Stasis can be resolved, especially in lower animals. The blood cells can again break loose and enter the blood stream as individual cells without any apparent damage. The same authors further state that the diagnosis of stasis can, with certainty, be made only by *in vivo* observations. In the ordinary histological prepa-

rations the smaller vessels, packed with blood corpuscles from stasis, cannot be distinguished from vessels filled with cells occurring in a dynamic hyperemia. The loss of cell individuality to the eye in the *in vivo* observations is reversed by the histologic technique. Shrinkage due to the dehydration breaks up the solid columns. Kreyberg and Rotnes (17) established, however, a technique whereby it is possible to diagnose stasis in preparations. If an experimental animal is injected with a vital stain before the freezing 'insult' is performed, one may, during the ensuing reaction (in this case during the thawing), observe how the area swells and takes the stain strongly. If later another stain is injected, this second stain cannot enter the area if stasis has developed. Likewise, if India ink is injected, the carbon particles cannot penetrate into the blocked vessels. In both cases the new injection will demonstrate an area uninvaded by the new stain. By varying the interval between the first and second injections, it is possible to find the moment when stasis develops, and by fixing the preparations and mounting them, it is possible to have permanent preparations, demonstrating the occurrence of stasis. The observations, and the technique of Kreyberg and Rotnes were practically unnoticed up to World War II.

Lange and Boyd (23) then adapted our technique for clinical use, by the introduction of fluorescein. This most beautiful method, where the dye is observed under ultraviolet illumination, shows a very strong staining during the period of thawing. In later stages, when stasis has developed, the damaged area stands out black against the stained surroundings. By photographic pictures it is possible to preserve permanent records of the different stages of this important vascular reaction. As to our observation of the development of stasis in connection with freezing, Lange and Boyd confirmed this completely. A most striking and convincing documentation is given by the microcinematographic film in colors, made by Quintanilla, Krusen and Essex (34) after freezing rabbit's tissue, using the Clark modification of Florey's window.

Stasis in itself means complete absence of circulation from the area involved, and this again involves necrosis. This was mentioned in our first paper (17), where we stated: "*La stase peut être temporaire ou permanente, et dans ce dernier cas détermine des troubles trophiques plus ou moins importants dans les tissus irrigués par les vaisseaux atteints.*"

As the finer mechanism is not fully known, and as the importance of this process was not generally accepted, a logical treatment for frostbite was impracticable during the recent war. Under the necessity of the time, however, several groups in different countries tried empirically to devise therapeutic interventions for preventing damage after freezing. The occurrence of clumping of the red cells had been observed many times previously, and this clumping played a great role in the considerations. Behind the gross picture of clumping, a series of different processes may be hidden.

Knisely and Bloch (12) described "intravascular agglutination of erythrocytes in disease," and stated, "a precipitate formed, which coated all the red cells, sticking them together in masses." In a later paper by Knisely, Eliot and Bloch (13) the term 'sludge' is introduced, and they state: "It seems reasonable to suspect that the 'sludge' initiator substances might be related to the substances capable of initiating blood clotting." Lange and Boyd (23) thought it promising to attempt to prevent



the formation of 'clots' by heparinization, subsequently to exposure. They performed such experiments and concluded that "heparin administered during the period of circulatory restoration prevented gangrene in 16 rabbits, whereas all controls had complete gangrene of the part."

Quintanilla, Krusen and Essex (34) did not confirm these findings. Crismon (6) states that the group at Stanford University were unsuccessful in their attempts to repeat Lange and Boyd's results, and Schumacker *et al.* (38) find it "is difficult to explain why in our experiments, with exposure of a smaller area for a shorter interval of time and with a more efficient method of anticoagulant therapy, the results were inferior to those reported by Lange and associates." Finally, also, Lange's group (9) agree, stating: "After thawing there are characteristic vascular engorgement and conglutination of red cells"; and further, "Our observations confirm those of Essex and Quintanilla (8) who found that heparin did not prevent early clumping of red cells within the vessels."

Summing up, there seems to be final agreement that heparin does not influence the development of stasis. This conclusion further supports the argument that the early clumping in stasis is not a process related to thrombosis. Moreover, since stasis in itself is a sufficient cause of necrosis, through ischemia, it seems possible to conclude that heparinization is of very little if any importance for the prevention of necrosis after freezing.

Agglutination is a process of clumping caused by specific substances of antibody nature, and the designation agglutination should be reserved for such processes. It would therefore be convenient to find other designations for the clumping of the red cells occurring in the early phases of stasis. Tannenberg and Fischer-Wasels (41) use the word 'conglutination', and the writer of the present review has tried to do the same, though with limited success only, because editors sometimes have changed the term during proofreading! A lack of terminological stringency evidently leads to confusion. The relationship between the two phenomena, conglutination in stasis and the 'sludge' formation of Knisely, is not yet established.

In the paper by Friedman, Lange and Weiner quoted above, these authors maintain their claim that "heparin prevents the development of gangrene after exposure to cold," and they find this "an observation which supports the view that thrombosis is important in the pathogenesis of the lesions." They further state: "Agglutinative thrombi, poor in fibrin, form and ischemic gangrene follows." The problem of necrosis is thereby linked up with another and later process than stasis. The 'agglutinative thrombi' mentioned by Friedman, Lange and Weiner are probably the same bodies which in the literature often are called 'hyaline thrombi', and described in connection with a great many pathological processes. Kreyberg and Vermés (18) discussed these intravascular masses and pointed out that these 'thrombi' in many cases actually are the necrotic blood cells after stasis. They are not precipitated or coagulated in a separate process, but they are simply the red corpuscles, trapped in the vessels during stasis, and undergoing necrosis parallel with the other tissue elements. They are not causing necrosis, but they are suffering necrosis as a part of the ischemic area.

Quite another question again is secondary thrombosis, which may develop in:

the vicinity of an area damaged by freezing, as in the surroundings of any damaged part of the body. This, however, is not a part of the cold problem per se, but a question of a secondary complication, like infection. That heparin may be of importance for the prevention of such secondary thrombosis is probable, but this question is outside the scope of the present review.

As previously stated, increased permeability is an important element in the development of stasis. Any means of controlling this factor may be of importance for the reduction of damage after freezing. Poulsson (33) made encouraging observations with 'pituitrin' in the control of edema after application of mustard oil to the eyes of rabbits, and Halpern's later experiments (10) with antihistamine substances may have a bearing upon the problem of reducing the damage caused by cold. Fuhrman and Crismon (9c) found that rutin delays the onset, but does not prevent stasis.

Ricker's theory (35) that special hydrodynamic conditions are of paramount importance for the development of stasis has been supported by our own observations. Recently Chambers and Zweifach (4) mention experiments with the injection of fever-producing toxins, where the arterioles become dilated while no change occurs in the normal rhythm of the vasomotion of the thoroughfare channels. This phenomenon is accompanied by hemoconcentration made evident by the close packing of blood cells in the collecting venules. A further study of the minute changes in the capillary bed may be of significance for learning how to control one factor of possibly great importance for the development of stasis.

That changes in the blood flow may be of importance for the development of necrosis after freezing has for a long time been acknowledged, even if the finer mechanism was obscure. Accordingly, a series of pharmacological and surgical interventions have been proposed and tried, very often based upon vague theoretical considerations, and belonging rather to the group of empirical explorations. Most of these studies concern the later stages of frostbite and are outside the province of this review.

Fuhrman and Crismon (9c) found that the following drugs and hormones were ineffective in the prevention of gangrene following frostbite. Vasodilator agents: carbon dioxide, nitroglycerine and acetyl-methyl choline chloride (Mechylol). Anticoagulants: heparin and dicumarol. Vasoconstrictors: epinephrine and/or synephrine. Steroid hormones: desoxycorticosterone and progesterone. Likewise alterations of blood volume, plasma colloidal osmotic pressure and extracellular phase volume by the use of whole blood transfusion, administration of concentrated human plasma albumin and intravenous injection of sodium chloride solutions, all failed to prevent gangrene following standard cold injury.

From purely empirical observations some observers have stated that rapid thawing at body temperature, or even higher, reduces the loss of tissue after freezing. An extensive review is given by Adams-Ray and Clemenson (1), and recent experiments reported by Fuhrman and Crismon (9a). The latter authors also present evidence that casts and pressure dressings are of importance (9b), even if Crismon admits that Lange and his group were not able to confirm the findings.

Natvig (31), in our laboratory, observed a considerably more destructive reac-

tion after X-ray irradiation on the sympathectomized, than on the control ear in rabbits. Accordingly, the present author made a series of experiments to study the effect of sympathectomy on the development of reactions after freezing (15). The main conclusions were that the vascular reactions to freezing after sympathectomy follow a different course from the reactions in the normal control ear. This difference is manifested by a more rapid and more abundant development of edema. The tissue reactions to the freezing varied in different individuals and in different areas of the same ear. The local changes leading to tissue necrosis in the frozen area were, with the doses in that experiment, mainly decided by the degree of the local insult. The tissue reactions were slightly more violent on the sympathectomized side, but the final damage was not altered, as measured with the technique used. Røden (37) froze rabbits' limbs and performed periarterial sympathectomy immediately after the termination of the exposure. He observed the same increase in general edema, and further, a permanent damage, which was not present in the control limb. These experiments give no support for the treatment of early damage due to cold by sympathectomy. The object there is not to increase but to decrease the vascular reaction.

The effects of local damage due to cold comprise a series of fundamental physiological and pathological reactions. The studies of recent years have added a number of observations, and at the same time shown the complexity of the problem. This will still for a number of years be a fascinating research field for the clinician, as well as for the pathologist.

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# REACTIONS OF BRITISH ANTI-LEWISITE WITH ARSENIC AND OTHER METALS IN LIVING SYSTEMS

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FOR an understanding of the nature of the action of arsenic on pathological micro-organisms such as *Treponema pallidum*, or of its toxic effects in man, it is clear that knowledge is needed not only of the nature of the biochemical defect which it produces in the life of the cell, but also of the chemical grouping or groupings with which it reacts. The problem is therefore chemical as well as biochemical in nature, and the identity of the 'chemo-receptors' for arsenic inside the cell has been sought since this chemical conception of the mode of action of arsenic was first given clear expression by Ehrlich (41).

The present review centers round the discovery during World War II of the pronounced antidotal properties of a dithiol, 2:3-dimercaptopropanol (British Anti-Lewisite, or BAL), against poisoning by a variety of arsenic-containing compounds, and, later, the extension of this work to poisoning by other metal compounds.

As these properties of dithiols were worked out with arsenic compounds in the first instance, it is proposed to trace the development of dithiol therapy from the early work relating the mode of action of arsenic to reaction with SH groups in the tissues of the body.

## BIOCHEMICAL LESION IN ARSENICAL INTOXICATION

Onaka (116) in 1911, working in Warburg's laboratory, seems to have been one of the first to attempt to bring the poisonous action of arsenic into line with specific functional effects produced upon the cells of the body. He showed that the addition of 0.0003 per cent sodium arsenite to nucleated red blood corpuscles caused a marked fall in their oxygen consumption, and suggested that the mode of action was identical with that of cyanide.

Dresel (32) next showed that the rates of respiration of certain rat tissues (liver, kidney and testis) and of Jensen sarcoma were powerfully inhibited by low concentrations of arsenite; with yeast both respiration and anaerobic glycolysis were found to be sensitive, although the anaerobic glycolysis of Jensen sarcoma was only slightly inhibited.

In 1930 Szent-Gyorgyi (172) attempted to analyze this effect of arsenite on cellular respiration, and demonstrated that it is not due to inhibition of the activation of oxygen by cytochrome oxidase. Using minced rabbit liver he stated that although the addition of 0.00013 M  $\text{As}_2\text{O}_3$  produced 59 per cent inhibition of respiration, the oxidation of *p*-phenylenediamine, which was entirely inhibited by low concentrations of cyanide, was quite insensitive even to 0.01 M arsenite. He also reported

that the oxidation of succinate is unaffected by 'high concentrations of arsenite.' In later work (4) a variety of different minced mammalian tissues was investigated; no specific dehydrogenase systems were studied, but it was concluded that arsenite brings about its effect on cell respiration by inhibiting the activation of hydrogen.

Voegtlin and his colleagues (186) next extended this work to cover the toxic effects of therapeutic arsenoxides. The influence of a series of pentavalent and trivalent arsenicals was examined, using various normal rat tissues, Jensen sarcoma and yeast. All the pentavalent arsenicals tested (arsenate, tryparsamide, 3-amino-4-hydroxyphenylarsonic acid and 4-hydroxyphenylarsonic acid) were found either to exert no influence on the oxygen consumption or to produce only a slight inhibition. The trivalent arsenoxides (e.g. 'mapharsen,' the hydrochloride of 3-amino-4-hydroxyphenylarsenoxide) on the other hand produced a substantial inhibition of all the systems studied both in the presence and absence of glucose. These results are of considerable interest in relation to the pharmacological action of arsenicals as studied by Voegtlin and Smith (187, 188). It was concluded by these workers that the arsenious oxides ( $\text{RAsO}$ ) are to be regarded as the active form of arsenic, and that the arspenamines ( $\text{RAs-AsR}$ ) and the pentavalent compounds ( $\text{RAsO}_2\text{H}_2$ ) must first be converted inside the animal body by oxidation or reduction into the active  $\text{RAsO}$  form. This biochemical transformation is carried out slowly, as is shown by the latent period of several hours in the trypanocidal action and the time of appearance of toxic symptoms in the host produced by these compounds; since the tissue-respiration experiments lasted only two hours, insufficient time was evidently allowed for the reduction of the pentavalent arsenicals, and there was consequently little or no effect on oxygen consumption.

Crasnaru and Gavrilesco (26), however, have claimed that novarsenobenzene (3:3'-diamino-4:4'-dihydroxyarsenobenzene-N-methylene sulphinate) does diminish the respiration of rat brain tissue, although exerting no inhibitory action on the respiration of muscle.

More recently the effects of arsenic on specific enzyme systems have been examined. In 1933, Krebs (84) put forward the view that arsenite is a specific inhibitor for the oxidation of  $\alpha$ -keto acids; his only apparent evidence for this was obtained in the course of experiments on the deamination of amino-acids. He found that rat kidney slices can rapidly bring about an oxidative deamination of amino-acids, and that in the presence of 0.001 M arsenite the corresponding  $\alpha$ -keto acids accumulate. Later Krebs and Johnson (85), showed that M/100 arsenious oxide inhibits the oxidation of pyruvic acid to acetic acid, lactic acid and carbon dioxide. These workers (86) also used arsenite to demonstrate the formation of  $\alpha$ -keto-glutarate during the oxidation of citric acid by pigeon breast muscle, while Thomas (175) used it to allow the accumulation of pyruvic acid formed from various intermediaries by pigeon muscle. In 1935 Jowett and Quastel (80) found that 0.001 M arsenite depresses both the oxygen consumption and the formation of acetoacetate from fatty acids by guinea-pig liver, and Quastel and Wheatley showed that  $2.5 \times 10^{-4}$  M arsenite inhibits the breakdown of acetoacetate by guinea-pig kidney (129). Potter and Elvehjem (127) studied the influence of M/300 arsenite on the oxygen uptake of yeast in the presence of a series of substrates, and obtained an almost complete inhi-

bition of oxidation of glucose, lactate and pyruvate. In the following year they (127a) studied the effect of arsenite on the succinoxidase system of chicken kidney, and, contrary to the earlier findings of Szent-Gyorgyi (172), claimed that this system was also sensitive to arsenite.

Using the methylene blue technique, Das (29) claimed that 0.125 M arsenite causes marked inhibition of both the lactate and malate dehydrogenases of pigeon breast muscle; Green and Brosteaux (64), however, had shown that more than twice this concentration had no effect on the lactate dehydrogenase of pig-heart muscle, while Green (63) found that a 0.03 M concentration actually caused an activation of the malate enzyme.

In 1941 Ochoa (113) showed that the fumarate-catalysed oxidation of pyruvate by brain dispersions, and also the accompanying phosphorylation of glucose, are inhibited by low concentrations of arsenite. He concluded that the inhibition of phosphorylation is secondary to that of pyruvate oxidation, since 0.008 M sodium arsenite has no effect on the phosphorylation of glucose by transfer of phosphate from phosphoglyceric acid.

The actions of sodium arsenite and some other arsenicals on a small number of enzymes other than those concerned in the oxidation-reduction mechanisms for carbohydrates and related substances have also received some attention: urease (137); maltase (136); pepsin (155); cathepsin (103); liver nuclease (105); amine oxidase (128); serum lipase (78); choline esterase (104) and mucinase (135) have all been investigated.

In a recent publication Gordon and Quastel (59) have reported that organic arsenoxides of the type  $\text{RASO}$  are inhibitors of choline dehydrogenase, and have confirmed the earlier finding that the oxidation of pyruvate, and possibly of other  $\alpha$ -keto acids, is markedly arsenic-sensitive.

From this brief survey of scattered observations in the literature it is clear that a considerable number of animal enzymes are arsenic-sensitive. At the same time there are no reports of any attempts to define by comparative measurements the specificity of the attack of arsenic upon living cells in terms of the relative sensitivity of different essential enzyme systems.

When work on the mode of action of arsenic was resumed under Peters's direction in 1939 it was decided that this was one of the first questions to attempt to answer. His earlier work (120, 124) had shown that iodoacetate selectively inhibits pyruvate oxidation in brain tissue, and in 1936 Peters (117) reported that traces of arsenite exert a similar effect. Using both sodium arsenite and  $\beta$ -chlorovinylidichloroarsine (lewisite), a number of enzyme systems related to carbohydrate metabolism were studied by Peters *et al.* (121) in order to map out their relative sensitivity to inhibition by these arsenicals. Of the enzymes studied the pyruvate-oxidase system proved outstandingly the most sensitive both in brain and also in skin (176), a tissue which was at the time of particular interest on account of the problems of lewisite vesication. The succinate, lactate and  $\alpha$ -glycerophosphate dehydrogenases, for example, were hardly affected by concentrations (0.034 mM.) of arsenicals which produced an almost complete inhibition of the pyruvate-oxidase system of brain. It was further concluded from this work that these arsenicals were reacting with a pro-

tein component of the pyruvate-oxidase system and not with any of the co-enzymes concerned with this system, as represented by cozymase, cocarboxylase and flavine-adenine dinucleotide.

The neurological manifestations which occur in certain cases of chronic arsenical intoxication are in many respects similar to those of vitamin B<sub>1</sub> deficiency (150), and it is of interest that *in vitro* evidence has also been obtained pointing to a similarity in the underlying biochemical derangement occurring in the two conditions, although operating at different points: an absence of an essential co-enzyme in the case of the latter, and a presumed inactivation of an essential protein in the former. In advanced vitamin B<sub>1</sub> deficiency *in vivo* evidence of the defect in pyruvate metabolism is shown by the marked elevation of the level of pyruvate in the blood (179). A similar raised blood-pyruvate level has been shown to be present in animals during either acute or chronic poisoning with sodium arsenite or after heavy contamination of the skin with lewisite (121). In man also, it has recently been reported that the blood-pyruvate level is elevated during arsenical intoxication (146).

These findings are in harmony with the earlier statements that arsenical poisoning is accompanied by derangements of carbohydrate metabolism. Oelkers (114), for example, had shown in 1939 that a rise of blood sugar occurs in rabbits within one hour of the injection of toxic amounts of inorganic or organic compounds of arsenic.

From the functional point of view, therefore, it would seem that inhibition of pyruvate oxidation in the tissues is one of the early and outstanding biochemical changes produced in arsenical intoxication. Moreover, from a consideration of the central position which pyruvic acid is now known to occupy in the metabolism of the cell (119) it is not surprising that a failure of its normal oxidation leads to far-reaching results.

#### CHEMICAL REACTIONS OF ARSENIC WITH CELL CONSTITUENTS

From the relatively small number of enzymes which have been studied in detail it would appear that the action of arsenic has a certain similarity to that of iodoacetate; thus, it has been shown (120) that the pyruvate-oxidase system of brain is sensitive to this poison at a concentration which leaves the lactate and  $\alpha$ -glycerophosphate dehydrogenases almost unaffected (75). The unpurified succinoxidase in muscle is also relatively insensitive to the action of iodoacetate (31).

Since the demonstration by Dickens (30) in 1933 that iodoacetate combines with the thiol groups in cysteine and reduced glutathione, results published by many authors have led to the view that whenever iodoacetate inhibits the activity of a biological system it does so by reaction with thiol groups present in that system and necessary for its normal function. In support of this view, Rapkine (131a) has shown that iodoacetate will also react with the thiol groups in proteins. Michaelis and Schubert (110) and Schubert (145) have further shown that although iodoacetate will combine with amino groups the rate of combination is very much slower than with thiol groups.

If this similarity between the actions of iodoacetate and the arsenicals on respiratory enzyme systems is real it might therefore be expected that arsenic also exerts its toxic action by combining with the thiol groups in certain enzymes, and it was



demonstrated early in the war by Peters and Wakelin (125) and later by Barron and Singer (9) that thiol groups are present in the pyruvate-oxidase system, and are essential for its functioning. From the previous work of Peters (118) on the toxic effect of diethyldichlorosulphone on the pyruvate-oxidase system and on its ability to combine with thiol groups, a connection had been established between the inactivation of an enzyme, vesication and reaction with SH groups.

As early as 1909 Ehrlich (41) had suggested, from theoretical considerations, that the 'chemo-receptors' for arsenic might be hydroxyl or thiol groups. Experimental evidence to support this speculation was later brought forward by Voegtlin and his associates (184). In 1923 they were able to show that the trypanocidal action of 3-amino-4-hydroxyphenylarsenoxide ('arsenoxide' or 'mapharsen') could be counteracted with the addition to the trypanosome suspensions of cysteine, reduced glutathione or other simple monothiols. About the same time, Walker (189), working under Peters's direction, found that the addition of di-substituted arsenicals, such as diphenylchloroarsine, to dried muscle powders (74) containing so-called 'fixed' SH groups (i.e., thiol groups in protein molecules) led to the inhibition of certain catalytic effects shown by these preparations; this was traced to the rapid disappearance of the SH reaction of the muscle powder, and was considered to be related to the toxic action of the arsenical, as there was a marked correlation between capacity for abolition of the fixed SH reaction and degree of irritancy.

In Walker's earliest work there was no complete proof that the inhibition of the SH reaction was due to combination of the thiol groups with the arsenical; subsequently, after the development of his cyanide test (190) for disulphide groups, it became clear that after removal of the SH reaction by the addition of arsenicals no recovery was brought about by treatment with cyanide, suggesting that a combination might have taken place.

It was further shown by Voegtlin *et al.* (185), that the toxic action of arsenoxide in rats could be diminished or prevented by the intravenous injection of glutathione immediately before the administration of the arsenical. In both trypanosomes and rats however a large excess of glutathione was required to abolish the effects of the arsenoxide, a ratio of 10 moles of glutathione to 1 of arsenoxide being necessary, while in the case of sodium arsenite it was found that a ratio of 40:1 was required to abolish trypanocidal activity (138). These workers also showed (186) that the reduction in oxygen consumption of animal tissues (rat testis, liver and kidney) caused by arsenoxide could be prevented by the addition to the system of reduced glutathione in a ratio of 10 moles to 1 of arsenoxide; but again, when sodium arsenite was used instead of arsenoxide, 40 moles of glutathione were required, a ratio of 10:1 being entirely without effect.

More recently this work has been extended by Eagle (36), who has shown that glutathione is also capable of abolishing the anti-spirochaetal action of arsenoxide; in this case also a large excess of glutathione was required (GSH:As = 10:1); cysteine was found to be less effective, even 12 to 14 moles failing to abolish the activity entirely.

Schmitt and Skow (143) investigated the ability of monothiols to prevent the toxic action of arsenite on the medullated nerves of frogs. They found that while cysteine and reduced glutathione delayed the extinction of the nerve action potential

produced by arsenite, in no case were they able to prevent the eventual extinction of the action potential, regardless of the ratio SH:As. Nor was it possible by the addition of these monothiols to produce any recovery once the action potential had been abolished by arsenite. They also claimed that a large excess of cysteine (150:1) was unable to prevent the inhibition of respiration of nerve produced by arsenite.

In the early work Walker (191) demonstrated that sodium thioglycollate had an inhibitory effect on the toxic action of diphenylchloroarsine on *Glaucoma*, he later showed that if monothioethylene glycol was added at the end of 3 minutes to a culture that had received a 3-minute lethal dose of diphenylchloroarsine rapid revival of the organisms occurred, but was followed by death in 1 to 2 hours.

It seems clear, therefore, that under the various conditions used by these workers an excess of a simple thiol compound, such as glutathione, is able to protect, to a certain extent, a variety of biological systems against the toxic effects of arsenoxide, and to a lesser extent against the effects of sodium arsenite.

Voegtlin, Dyer and Leonard had, moreover, suggested that in addition to this inability to combine with simple thiols arsenic might also react with thiol groups present in proteins, and Rosenthal (139) obtained evidence that trivalent arsenic could not be ultra-filtered from suspensions of heat-coagulated proteins containing thiol groups, and that the nitroprusside reaction of these protein suspensions and of washed muscle powder and washed minced liver suspensions could be caused to disappear by treatment with arsenoxides.

In view of these findings, when work on the arsenical vesicants was actively taken up in 1939 it was clear that thiols should be further studied to discover whether any degree of protection was afforded by them against lewisite as had been shown to be the case with the therapeutic arsenicals. *In vitro*, lewisite had been found significantly more toxic to the pyruvate-oxidase system than either sodium arsenite or the therapeutic compounds, and when, using this system, simple monothiols were tested it was found that no protection was afforded against lewisite, even when the thiols were present in concentrations up to 200 times that of the lewisite (151).

With arsenicals of relatively low toxicity, therefore, excess of simple thiols can bring about a limited degree of protection, but when more toxic compounds such as lewisite are used all the thiols, which up to that time had been used, are ineffective even when present in large excess. From these results it seemed that further information was needed concerning the nature of the reaction between arsenicals of different types and thiol-containing proteins. Cohen, King and Strangeways (24) had already shown that different arsenicals behave *in vitro* in very different fashions towards thiol compounds. These authors, in addition to clearing up many of the anomalies which existed in the literature with respect to the nature of the reaction between arsenicals and thiols, established the fact that the equilibrium of the reaction  $\text{RAsO} + 2\text{R}'\text{SH} \rightleftharpoons \text{RAs}(\text{SR}')_2 + \text{H}_2\text{O}$  depends on the hydrogen ion concentration, acid shifting it to the right and alkali to the left. It can also be seen from their data that different thioarsinites undergo different degrees of hydrolysis at the same pH; for example, di(glutathionyl)-benzamide-*p*-thioarsinite was said to give a negative nitroprusside test in sodium bicarbonate solution, while di(carboxymethyl)-acetanilide-*p*-thioarsinite gave a feeble one.

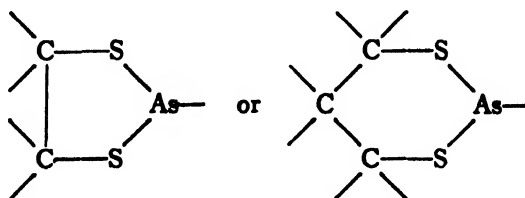
This work was continued and given greater biochemical importance by Strange-

ways (168), who studied the extent to which aromatic thioarsinites were dissociated under physiological conditions. She was able to show that the lethal activities of equimolar solutions of di(glutathionyl)-3-amino-4-hydroxyphenylthioarsinite and the parent arsenoxide were identical. Moreover, using the dilution of arsenoxide employed by Voegtlin *et al.* (184), she confirmed their observations that complete inhibition of the toxic action could be obtained with 10 mols. of glutathione, but found that on using more dilute solutions of arsenoxide the protection afforded by 10 mols. of glutathione became less complete; finally at high dilutions no protection was observed at all. In concentrated solution the excess of glutathione favours the formation of the thioarsinite, while in more dilute solutions the great preponderance of water molecules brings about hydrolysis with liberation of the toxic arsenoxide. Also relevant in this connection is the work of Voegtlin *et al.* (186) who stated that tricysteinyarsine, first prepared by Labes (88), was as toxic to the respiration of testis as the original arsenical.

From the later work on the reaction between arsenicals and kerateine described below it is now clear that the inability of the monothiols to protect against the more toxic arsenicals lies in the different degree of dissociability of the simple thioarsinites and the protein thioarsinites which are formed with cell constituents.

#### INTRODUCTION OF DITHIOLS AS ANTIDOTES

In studying in more detail the interaction between arsenicals and thiol-containing proteins it was first shown that when compounds such as  $\beta$ -chlorovinylarsenoxide ('lewisite oxide') react with the soluble protein kerateine under physiological conditions of hydrogen ion concentration and temperature, the arsenic content of the derived protein depends on and is directly related to the SH content of the original kerateine; further, the analytical figures showed that approximately 75 per cent of the arsenic present had combined in the proportion of 1 As: 2 SH (164). Since the protein thioarsinite was comparatively stable to dialysis and since kerateine readily undergoes oxidation to the disulphide form, it seemed possible that the arsenic had combined with two SH groups closely placed on the same molecule to form a large, relatively strainless ring. From these considerations it followed that, in order to displace arsenic from its cyclic combination with proteins such as kerateine, the presence of competing dithiols, which could form even more stable cyclic compounds than the protein, would be necessary. The most likely dithiols were those containing 1:2 or 1:3 substitution, which would therefore give rise to five- or six-membered rings of the types:



A number of dithiol compounds were therefore prepared and put to the test as possible antidotes for lewisite. An initial chemical justification for the ring hypothesis men-

tioned above was obtained by showing that the relative stabilities of monothiol-thioarsinites, protein-thioarsinites and dithiol-thioarsinites increased in this order. Further, *in vitro* experiments with the pyruvate-oxidase system showed that dithiols were not only able to protect against the inhibitions produced by lewisite and other arsenicals, but also reversed the toxicity, regenerating the enzyme in its active form, after a period of poisoning by the arsenical (165).

For the application of dithiol therapy to man, 2:3-dimercaptopropanol was selected from the other dithiols which had at that time been studied in this connection. This choice was made on account of its lower toxicity, and because its physical properties were suitable for the purpose then in view, namely the treatment of skin contaminated with lewisite.

BAL was therefore first tested for its ability to bring about the survival of rats and guinea-pigs after the application of lethal doses of lewisite to the skin, or the injection of lethal doses of sodium arsenite. It was also shown that, in addition to its ability to cause the survival of such animals, treatment with BAL caused an increased elimination of arsenic in the urine (166). This finding has since been studied in some detail in man (*vide infra*).

In search of further evidence in support of the 'ring hypothesis' of arsenical toxicity which developed in the course of this work, Whittaker (195) synthesized a series of  $\alpha:\omega$ -dithiols from ethane to dodecane-dithiol and compared their relative antidotal efficiencies. In agreement with the existing views regarding the ease of formation of cyclic compounds of different ring sizes he found that dithiols forming 5- or 6-membered rings with the arsenical were also the most effective antidotes.

The comparison of mono- and dithiols in the protection of enzyme systems from inhibition by lewisite has also yielded results suggesting that certain enzymes may themselves be regarded as mono- or dithiol in type (178). The different sensitivity of two 'SH enzymes', the pyruvate-oxidase system and the succinate dehydrogenase, has already been referred to. Also, while no monothiol has yet been found which, under the conditions used by Peters *et al.* (121), is capable of protecting effectively the pyruvate system, cholinesterase, which is also sensitive to arsenic, (104, 177) can be protected almost equally effectively by either mono- or dithiols. These two sets of facts suggest that the pyruvate oxidase system contains a 'dithiol enzyme' whereas cholinesterase is a 'monothiol enzyme'.

#### CHEMISTRY OF THE DITHIOLS

The very considerable amount of work carried out during the war on the preparation and production of dithiols has not as yet been fully described in the literature, and in consequence this review cannot do justice to all the workers concerned.

The aliphatic dithiols are most satisfactorily prepared from the corresponding halogen compound and sodium or ammonium hydrosulphide. The reaction is carried out in alcoholic solution, and in order to avoid gross contamination of the product with complex sulphides it is essential to employ a closed system (162, 77). It is of interest to note that, subsequent to the work in Oxford, Sjöberg (152) independently prepared 2:3-dimercaptopropanol by means of potassium thioacetate, but the yield obtained was significantly lower. Thioacetate is of more value in special circum-

stances, and was employed with success by Ellingboe, Pavlic, Signaigo and Lazier (42) for the preparation of 2:3-dimercaptopropionic acid, and by Whittaker (195) for 1:4-butane dithiol. One further method for the production of vicinal dithiols was devised by Meade and Woodward (109) who obtained ethane dithiol by the addition of  $\text{H}_2\text{S}$  to ethylene sulphide. So far as we are aware all other reagents which lead to monothiols fail when applied to the preparation of dithiols.

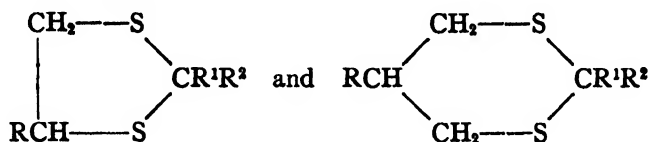
Although BAL can be distilled *in vacuo* in comparatively small quantities, on the large scale it is advantageous to add ammonium salts (52), or carboxamides (132), as stabilizers. Most of the dithiols are more soluble in fat solvents than in water, and attempts have been made to prepare water-soluble derivatives possessing equal therapeutic efficiency and lower toxicity. By condensation with methylol derivatives of secondary amines or amino-acids Kharasch and Weinhouse (82) obtained com-

pounds such as  $\begin{array}{c} \text{CH}_2\text{SH} \\ | \\ \text{CHS} \cdot \text{CH}_2\text{N R}^1\text{R}^2 \\ | \\ \text{CH}_2\text{OH} \end{array}$  which although highly water soluble are of less value in other respects.

More recently Danielli *et al.* (28), prepared BAL-glucoside (BAL-Intrav),  $\text{CH}_2\text{SH} \cdot \text{CH SH} \cdot \text{CH}_2\text{OC}_6\text{H}_{11}\text{O}_6$ , a compound with very low toxicity and, on account of its water-solubility, more suitable for intravenous injection.

The dithiols condense with substituted dichlorarsines or arsenoxides to give derivatives of 1:3-dithia-2-arsacyclopentane,  $\begin{array}{c} \text{CH}_2-\text{S} \\ | \quad \diagup \\ \text{CH}_2-\text{S} \quad \text{AsR} \end{array}$  and 1:3-dithia-2-arsacyclohexane,  $\begin{array}{c} \text{CH}_2-\text{S} \\ | \quad \diagup \\ \text{CH}_2-\text{S} \quad \text{AsR} \end{array}$ , as shown by Stocken (162) and by Peters and Stocken, (123). Two compounds of the former class were earlier described by Cohen *et al.* (24).

With carbonyl compounds the dithiols form dithiolanes and dithianes.



A number of metal salts react with dithiols to give either intense colors or precipitates (159, 7), and the reaction with cobalt has been employed for the estimation of BAL (13, 159). The reaction with cadmium has been studied in some detail by Gilman *et al.* (55).

The effect of catalytic amounts of heavy metals on the oxidation of dithiols has been described by Barron *et al.* (7). These authors found that copper was the most powerful catalyst, and also showed that when iron porphyrins were used as catalysts the porphyrin ring was opened; in view of the immediate reduction of methemoglobin by BAL they suggested that the reaction could be utilised for the treatment of methemoglobinemia.

In the course of a study of the nephrotoxic action of tetrathionate Gilman and his associates (56) found that under *in vitro* conditions BAL was oxidized to the disulphide with simultaneous reduction of the tetrathionate to thiosulphate.

In addition to the cobalt reaction already mentioned other methods have been described for the estimation of dithiols. Barron *et al.* (7) have determined dithiols by spectrophotometric measurement at 6900 Å of the ferrocyanide formed as Prussian blue by reduction of ferricyanide. Aldridge (1) found that dithiols react with cyanogen chloride to produce unidentified compounds which liberate thiocyanate on treatment with alkali, the estimation of the dithiol being thereby reduced to the estimation of thiocyanate, for which micro-methods are available. Chowdhury (in press) has recently proposed an enzymic method based on the protection of urease against the toxic action of mercuric chloride. In conclusion, however, it must be stated that as yet there is no specific method for the estimation of vicinal dithiols in general or of BAL in particular.

Since BAL possesses an asymmetric carbon atom it would be of interest to know whether the optical enantiomorphs have different toxicities, and a considerable effort has been directed by Snyder (156) towards the resolution of BAL. Although he obtained the strychnine salts of the formaldehyde derivatives in an optically pure state the regeneration of the BAL proved unsuccessful.

#### PHARMACOLOGY AND TOXICOLOGY OF BAL

In view of the effectiveness of 2:3-dimercaptopropanol in preventing vesication of human skin by lewisite, even when applied as late as one hour or longer after contamination (165, 181), work was early undertaken on the pharmacology and toxicology of the compound.

The LD<sub>50</sub> dose of BAL for laboratory animals appears to be between 50 and 150 mg/kg. when administered parenterally. When dissolved in propylene glycol and injected intramuscularly into white rats Stocken and Thompson (165) obtained an LD<sub>50</sub> of 113 mg/kg. Graham (60), however, has recently shown that quite large sub-lethal doses may be given to young rats repeatedly with little if any evidence of toxic effects, and without effect on appetite or rate of growth. The doses he used were 10 mg/kg. for periods up to 56 days, and 50 mg/kg. up to 14 days. The thyroid glands were slightly heavier in the injected rats than in the controls, indicating a slight degree of goitrogenic activity; no evidence of liver or kidney damage was detected. Using dogs, Macnider (98), on the other hand, produced fatty lesions of the liver by administering doses of 20 mg/kg. twice daily for 4 days, although doses of 5 mg/kg. were tolerated well.

The mortality rate of rats injected with BAL has been shown to be markedly influenced by the temperature of their environment, the mortality being minimal at 17.2°C. (107).

Chenoweth (19) carried out a study of the action of 2:3-dimercaptopropanol on the cardio-vascular system. He showed that the intravenous or percutaneous administration of small doses to cats caused a rise in blood pressure. Larger doses, however, produced signs of capillary damage and of peripheral vascular failure. It also caused a striking increase in the rate of lymph flow from the cervical and thoracic

ducts. In perfusion experiments with isolated organs Hitchcock (72) demonstrated that BAL produces a reversible constriction of the vessels of the cat's limb.

Krop (87) studied the actions of BAL on the isolated sartorius muscle of the frog, and was able to show that in concentrations of from M/500 to M/2000 it caused a marked reduction or complete abolition of the oxidative heat associated with the contraction process. He also found that lactate accumulated in the fluid bathing the muscle.

The toxic effects in animals of large doses of BAL have been studied by Durlacher *et al.* (34). Toxic but sublethal doses of BAL given to mice, rats, guinea-pigs, rabbits and dogs, cause lacrimation and blepharospasm. In dogs salivation, retching and vomiting also occur. When still larger doses are administered muscle tremors develop, which may increase in intensity and become replaced by generalized tonic and clonic convulsions, together with a loss of co-ordination and ultimately coma. Once convulsions have developed it is rare for an animal to survive. The muscle tremors and convulsions are not the result of hypoglycemia, since it has been shown that the blood sugar is normal in animals exhibiting these signs, although shortly before death it may fall to hypoglycemic levels. Large doses of BAL produce a metabolic acidosis, as is shown by a fall in the pH and CO<sub>2</sub>-combining power of the blood together with an increased serum-lactate level; the blood amino nitrogen is also raised, while the liver glycogen content and liver potassium-level are lowered.

Similar toxic symptoms are also produced in cats as noted by Modell *et al.* (111), the earliest signs of poisoning being blinking, blepharospasm, lacrimation, conjunctival edema and salivation. Many of the toxic actions of BAL are, however, reversible; the above workers have shown, for example, that animals severely poisoned with  $\frac{1}{3}$ LD<sub>50</sub> dose may be symptom-free in about 5 hours.

Chenoweth, Modell and Riker (20) and Fitzhugh *et al.* (47) have also reported on the pharmacological properties and toxicities of a number of thiols related to 2:3-dimercaptopropanol. Although in general many of the toxic signs produced in experimental animals closely resembled those due to BAL, a number of distinct and interesting differences in the response of the animals was observed as a result of only minor changes in the structure of the thiol.

An 'explosive' reaction characterised by motor and parasympathetic overactivity has been described in rabbits after the administration of sodium arsenite and BAL (83), but the manifestations of this reaction were short-lived, and none of the affected animals died.

A detailed study of the effects produced in man by the injection of BAL was carried out by Modell, Gold and Cattell (112). These workers injected a 10 per cent solution of BAL in peanut oil containing 20 per cent benzyl benzoate intramuscularly in single doses ranging from 3.0 to 8.0 mg/kg. and in repeated doses totalling from 8.0 to 20.0 mg/kg., these amounts being given in from 8 to 10 hours. They found that the minimum dose required to produce toxic symptoms was between 3 and 5 mg/kg. As in the work with animals, lacrimation, blepharospasm and salivation were observed. Nausea, vomiting, paresthesia (burning and tingling) of nose, eyes, mouth and skin and pain (principally in the limbs, jaws, abdomen or head) also occurred. The blood pressure, both systolic and diastolic, was raised and the pulse

rate increased. Symptoms appeared rapidly after the injections, but usually disappeared with two hours. Observations on subjects given repeated injections led to the conclusion that doses of up to 5 mg/kg. can be given at 3-hourly intervals without evidence of any cumulative action. Very similar results were obtained by Sulzberger, Baer and Kanof (169), who also studied the percutaneous toxicity to man and showed that more than 1 gm. of BAL, made up in a 3 per cent jelly, can be applied to large areas of skin without producing any signs of systemic poisoning; locally, the application of BAL or of 5 or 10 per cent BAL ointments produces in most subjects an evanescent erythema, and in some cases an urticarial reaction. It has been claimed that the unpleasant side-effects which sometimes accompany the intramuscular injection of BAL can be prevented by the administration of adrenaline or ephedrine (183).

In a further paper Sulzberger, Baer and Kanof (170) showed that the daily inunction of BAL into the skin for 14 days can produce a low-grade sensitization dermatitis; in all cases, however, the eruption subsided without treatment in less than 6 days after the last application. Repeated intramuscular injection of BAL also gave rise to a sensitization, as shown by the development of positive patch tests.

A study of the tolerance to BAL of animals with experimentally produced impairment of hepatic and renal function has been carried out by Cameron, Burgess and Trenwith (14). They showed that animals with renal damage showed the same tolerance as normal animals and it was only when complete or almost complete failure of renal function was present that some lowering of tolerance was observed. Animals with hepatic damage, however, did show toxic signs, and in some instances died after receiving amounts of BAL well below the lethal amounts for normal animals.

Turning to the biochemical effects produced by BAL, Webb and van Heyningen (193) showed that, with the exception of the cytochrome-oxidase system, BAL is an efficient inhibitor of metal-containing enzymes: polyphenol oxidase, carbonic anhydrase, catalase and peroxidase are all powerfully inhibited by concentrations as low as 0.001 M. Hexokinase, functioning in the presence of sub-optimal amounts of magnesium ions is also inhibited by BAL, this effect being readily reversed by the addition of excess  $Mg^{++}$ . According to these workers the cytochrome system is not inhibited by BAL, but, instead, brings about its rapid oxidation. Barron, Miller and Meyer (8) have also studied the effect of BAL on enzyme systems, and have in general confirmed the results of Webb and van Heyningen; in contrast to the latter's findings, however, Barron *et al.*, using a spectrophotometric method, have stated that the oxidation of reduced cytochrome *c* by cytochrome oxidase is inhibited by low concentrations of BAL. They also showed that the hypoglycemic effect of insulin in rabbits can be abolished by allowing the insulin to stand at room temperature in the presence of aqueous solutions of BAL; this inactivation is probably due to reduction of the —SS—groups of the insulin molecule.

Under *in vitro* conditions BAL delays the clotting of blood and is more active in this respect than either cysteine or glutathione; this effect is brought about by inactivation of prothrombin, possibly by the reduction of —SS— groups in the molecule (44).

Since BAL is a powerful reducing agent it is capable of converting methemoglobin into hemoglobin, and it has been shown that it can reduce the degree of experi-



mentally produced methemoglobinemia in dogs (10), although it is not as effective in this respect as methylene blue.

#### METABOLISM OF BAL

In a preliminary study of the metabolism of BAL Stocken and Thompson (166) found that the injection of relatively large doses into rats or rabbits causes the excretion of a thiol (or thiols) in the urine; the amount of this thiol in the urine of rabbits appeared to reach a maximum at about 1-3 hours after the injection of a single dose of BAL. Its excretion was occasionally accompanied by a transient albuminuria, but no evidence was obtained of any serious or permanent functional damage to the kidney even after these large doses.

Using a colorimetric method (13, 157) based on the formation of the cobalt mercaptide, Spray *et al.* (159) obtained further evidence suggesting that the excreted thiol is closely related to BAL, but is not identical with it. The fact that it is capable of protecting the brain pyruvate-oxidase system from inhibition by lewisite at concentrations at which no monothiol has yet been found to be effective suggests that the derivative of BAL which is excreted in the urine is also a dithiol.

A sharp rise in the urinary excretion of sulphur occurs in the first 24 hours after injection of BAL into rats; the 'ethereal sulphur' fraction of the urine is however unaffected, suggesting that, in the rat, detoxication of BAL does not occur by means of conjugation with sulphate (158). Peters *et al.* (122), using radioactive BAL ( $^{35}\text{S}$ ), extended these findings and showed that the sulphur of BAL is largely excreted in the 'neutral sulphur' fraction. They also studied the distribution and persistence in the circulation of  $^{35}\text{S}$  after injection of radioactive BAL into rats. Kidney, small intestine and liver were found to contain considerably more  $^{35}\text{S}$  than other organs. Similar results with radioactive BAL have been obtained by Simpson and Young (149) for the distribution at a rather longer time interval.

The evidence so far obtained suggests that BAL is fairly rapidly removed from the blood stream. Peters *et al.* (122) showed that  $^{35}\text{S}$  remained in relatively high concentrations in the blood for 1 to 2 hours after injecting radioactive BAL, although the technique employed in these measurements did not differentiate between BAL itself and its metabolic products. Most of the observable pharmacological and biochemical effects of a single dose of BAL disappear within about 4 hours, and in the administration of repeated doses of BAL an interval of this order has been suggested between successive doses in an attempt to keep up the concentration of the drug in the blood.

#### THERAPY OF EXPERIMENTAL ARSENICAL POISONING WITH BAL

The original work on the treatment of lewisite intoxication in rats and guinea-pigs by 2:3-dimercaptopropanol by Stocken and Thompson (165) has already been mentioned, and as an extension to this work a small number of experimental lewisite contaminations on the forearms of human subjects was also carried out and treated with BAL. The ineffectiveness of monothiols in the decontamination of lewisite on human skin was confirmed by Thomson *et al.*, (181), who also tested a number of other dithiols, including several water-soluble derivatives of BAL, several of which proved highly effective decontaminants.

The early work on the protection and reactivation of enzymes from the effects of lewisite and sodium arsenite has been confirmed and extended by Barron *et al.* (6).

The therapeutic effect of BAL on tissue cultures poisoned with lewisite oxide was studied by Fell and Allsopp (45), who found that a 10:1 excess (w/v concentration) of BAL in the culture medium was sufficient to protect the cells from the action of a just sub-lethal concentration of lewisite oxide.

In animal experiments it has been shown that treatment of an area of lewisite-contaminated skin with BAL causes a significant reduction in the amount of arsenic remaining in the scar after 48 hours (166). Graham, Levvy and Chance (62) have also reported that BAL, applied directly to an area of contaminated skin, prevents or even reverses the fixation of arsenic in the skin, although the rate of absorption of arsenic was not noticeably increased.

The rate of excretion of arsenic in the urine is, however, significantly increased by BAL therapy; this has been shown in rats contaminated with lewisite (166), and in rabbits after the recent injection of either phenylarsenoxide or mapharsen (18).

In extension of the earlier work with sodium arsenite and the chemical warfare arsenicals, Stocken, Thompson and Whittaker (167) showed that BAL can also protect the brain pyruvate-oxidase system from the inhibitory actions of mapharsen, arspenamine and neoarsphenamine, and can also bring about the survival of rats after the injection of lethal doses of these arsenicals. In this connection, however, it is important to note that Peters and Stocken (123) have found that the compound formed by BAL and mapharsen is more toxic than mapharsen alone when injected intramuscularly into rats; in the presence of one mole excess BAL, however, the mapharsen-BAL complex has only about one fifth the toxicity of mapharsen. The same authors (123), Friedheim and Berman (49) and Friedheim and Vogel (50) have shown that *in vitro* this compound more readily immobilizes various strains of micro-organisms than mapharsen. Ercoli and Wilson (43) investigated the effect of increasing amounts of BAL given in conjunction with mapharsen. They concluded that the curative action of the arsenical against *T. equiperdum* in mice was more readily affected than the trypanocidal activity, and that this method of treatment had no chemotherapeutic value.

Eagle, Magnuson and Fleischman (40) have carried out an extensive study of the treatment with BAL of experimental mapharsen poisoning in rabbits. In the course of this work they developed a stable solution of BAL in arachis oil and benzyl benzoate which was capable of sterilization by heat (160°C. for 1 hr.), and which has formed the basis of all the preparations subsequently used therapeutically in man. They showed that intramuscular injections of this sterile solution of BAL proved effective in the treatment of mapharsen, lewisite and phenylarsenoxide poisoning in rabbits. Eagle and his colleagues (194), also studied the effect of BAL on the excretion of arsenic in man after exposure of subjects to an arsenical smoke (diphenylcyanoarsine). As in the work with animals already described, they found that a single intramuscular injection of BAL produced a significant and regular increase in the rate of arsenic excretion in the urine of both normal subjects and those exposed to the arsenical.

In cats acutely poisoned with mapharsen Riker (133) found that intramuscular injection of BAL dissolved in cotton-seed oil gave complete protection, and that this

oily solution was more effective in saving life than solutions of BAL in saline. He also reported on 2:3-dimercaptopropyl ethyl ether, which, however, he found considerably less effective as an antidote against mapharsen, although apparently effective in preventing the hemolytic changes associated with arsine poisoning.

A study of the effect of BAL on the blood and plasma concentrations of arsenic in cats poisoned with mapharsen has been carried out by Riker and Rosenfeld (134); they reported that a single dose of BAL rapidly causes a sharp rise in the whole blood and plasma levels of arsenic, and conclude that since this rise is chiefly in the plasma fraction, it reflects an increased rate of mobilization of tissue arsenic prior to its urinary excretion.

Further evidence of the value of BAL in the treatment of experimental lewisite poisoning in dogs was obtained by Harrison *et al.* (67, 68).

A therapeutic application of considerable urgency and importance at the time of the discovery of BAL was its possible use in the treatment of eyes contaminated with lewisite vapor or droplets. Its value in this connection was demonstrated independently by Mann, Pirie and Pullinger (101) and Hughes (76). Briefly, it was found that the destructive action of lewisite on the rabbit's eye can be prevented by the instillation of a 5 per cent BAL solution or ointment into the conjunctival sac within 5 minutes of contamination, while a distinct improvement in the ultimate condition of the eye was noticed even when treatment was delayed for as long as 20 minutes after contamination.

In addition to Riker's (133) findings with 2:3-dimercaptopropyl ethyl ether already mentioned, Levvy (91) has reported on the use of ethane-1:2-dithiol in arsine poisoning, and Kensler, Abels and Rhoads (81) have studied the ability of a number of mono- and dithiols to prevent the lysis of erythrocytes by arsine *in vitro*; several dithiols were also effective in protecting tissue respiration from inhibition by arsine, and in saving the lives of rabbits, dogs and monkeys.

#### CLINICAL USE OF BAL IN ARSENICAL INTOXICATION

The application of 2:3-dimercaptopropanol to the treatment of the complications that may develop in the course of arsenotherapy in man followed rapidly on the demonstration of its value in experimental poisoning with lewisite or sodium arsenite, and a very considerable amount of information is now available on this aspect of its uses.

The first cases of widespread arsenical lesions in man to be treated with BAL received the compound by inunction of 5 or 10 per cent ointments. Longcope *et al.* (95) treated 7 cases of dermatitis occurring in workers exposed to phenarsazine chloride and 4 cases of exfoliative dermatitis produced by anti-syphilitic arsenicals; highly satisfactory results were obtained, and it was observed that the inunction of the BAL ointment into unaffected areas of skin produced as rapid clearing of the dermatitis as when it was rubbed into the inflamed areas themselves. This was, at the time, a point of some practical importance owing to the intense though transient burning sensation brought about by application of the ointment to inflamed skin. These workers also treated a further 11 cases of exfoliative post-arsphenamine dermatitis with intramuscular injections of 5 or 10 per cent BAL in peanut oil and benzyl benzoate, and obtained symptomatic and objective improvement. Carleton and asso-

ciates (15) also reported an early series of 30 cases of arsenical dermatitis, mostly following injections of neoarsphenamine; 9 of these cases were treated by inunction, and the remainder by injection. A careful analysis of the clinical results indicated a beneficial effect in a substantial number of cases.

A further series of 44 cases has been described by Carleton, Peters and Thompson (16); in this series a 'good' or 'fair' response was obtained in 31 cases (70%). A report from the BAL Conference (126), convened by the Medical Research Council, has appeared, based on the results obtained in these 44 cases and on other publications then available.

In most cases of acute exfoliative dermatitis the edema of the skin and the oozing, if present, are among the first signs to show a response to BAL therapy, their decline usually occurring within 2 to 3 days of the first injection. A course of injections is usually given, lasting for 5 or 6 days, and it is not uncommon for a slight relapse to be observed some time after the last injection; such relapses have, however, usually responded well to a further few injections.

In addition to arsenical dermatitis BAL has been used with success in the treatment of a number of other complications of arseno-therapy. Eagle and Magnuson (39) and Eagle (37) have reported on its use in arsenical encephalopathy and agranulocytosis and in patients who in error had received massive doses of mapharsen; their results in so-called arsenical jaundice were unsatisfactory, but there is reason to believe that in many of these cases the immediate cause of the hepatitis is infective (141). Byrne (12) and Csonka (27) have also described cases of encephalopathy treated with BAL, while Holley (73) and Schrumpf (144) have reported on its use in arsenical granulocytopenia and thrombopenic purpura respectively. Woody and Kometani (196) have treated 42 children suffering from acute arsenical poisoning due to ingestion of substances containing arsenic, and state that in comparison with a previous group of 111 cases not treated with BAL the toxic symptoms subsided sooner and less hospitalization was necessary. It is of interest to note that although doses of 25 mg. BAL/kg. or more were given no fatalities due to the toxicity of BAL occurred.

Friedenberg (48) has reported the successful use of BAL in the treatment of optic neuritis occurring in a patient with tabes dorsalis who had received two small doses of tryparsamide.

Some conflicting results have been obtained regarding the effect of BAL on the urinary excretion of arsenic in arsenical intoxication in man. Luetscher, Eagle and Longcope (96) found a definite increase in arsenic excretion during the treatment of cases of dermatitis. Carleton *et al.*, (15), however, concluded that BAL had no demonstrable effect on the urinary elimination of arsenic in these cases. The day-to-day variations in arsenic excretion even in the absence of BAL are marked, and the different findings of these workers may also have been influenced by the state of acuteness of the intoxication, and the interval between the last injection of the arsenical and the start of BAL therapy.

#### TREATMENT OF OTHER METAL INTOXICATIONS WITH DITHIOLS

**Mercury.** It has been known for a number of years that compounds of mercury react with essential SH groups in enzymes (131, 71, 36), and in 1940 Fildes (46)

showed that the inhibitory effects of mercuric chloride on the growth of *Esch. coli* could be prevented and reversed by the presence of monothiols such as glutathione or cysteine.

In view of the ability of 2:3-dimercaptopropanol to form non-ionized complexes with heavy metals other than arsenic, the use of dithiols as antidotes in cases of poisoning by mercury was soon initiated. One of the earliest reports in this connection was the description by Longcope and Luetscher (94) of 23 acute cases of mercuric chloride poisoning treated with intramuscular injections of BAL; 22 of the 23 patients studied recovered completely, although the amounts of mercury swallowed varied from 0.5 to 20 gm.

More recently, a number of detailed studies have been made of the ability of monothiols and dithiols to protect against and to reverse the toxic action of various mercury compounds. Barron and Singer (9) have reported that glutathione is able partially to reverse the toxic effect of *p*-chloromercuribenzoic acid on the pyruvate-oxidase system of gonococci, and Barron and Kalnitsky (5) have found that various dithiols can bring about from 60 to 85 per cent reactivation of pigeon breast muscle succinic oxidase after poisoning with mercuric chloride. In the case of the pyruvate-oxidase system of brain Thompson and Whittaker (180) found that both BAL and glutathione were able to protect against the effects of  $\text{HgCl}_2$ .

The tissue-culture technique has been used by Harrison and Randoll (69) to compare the relative merits of BAL, BAL-Intrav and sodium sulphide in preventing the inhibition of growth by mercuric chloride. Although BAL was of some value, it was stated that the two other compounds were more effective, and approximately equally so.

In a study of the reaction of the isolated heart to the organic mercurial Salyrgan, Long and Farah (93) showed that either mono- or dithiols could prevent the toxic manifestations, but in the intact dog BAL was effective at one-fifth the molar concentration of the monothiols. They also investigated the mortality rates in mice dosed with Salyrgan after a previous injection of either cysteine, glutathione or BAL, the mercurial and the thiol being both administered intravenously; glutathione and BAL were found to have approximately equal antidotal effects, while cysteine was rather less active.

Confirming the clinical observations of Longcope and Luetscher (*vide supra*), it has been shown in animal experiments that BAL can effectively counteract the acute toxicity of mercuric chloride. Gilman *et al.* (54) administered mercuric chloride intravenously to rabbits and dogs, while Stocken (161), working with rats, gave the mercurial intraperitoneally. These investigations also showed that BAL possesses considerable antidotal effects against orally-administered  $\text{HgCl}_2$  in dogs and rats. Braun, Lusky and Calvery (11) have also reported briefly on the use of BAL in  $\text{HgCl}_2$  poisoning in rabbits, while Graham and Hood (61) have shown a protective action in mice.

Further evidence of the antagonism between BAL and mercury compounds is provided by the work of Handley and La Forge (65), who found that in dogs the diuretic action of Mercuhydrin [1-(methoxy-oxymercuripropyl)-3-succinyl urea] is abolished by BAL, but is only partially diminished by monothiols. In seven cases

of congestive heart failure Sussman and Schack (171) observed a complete annulment of the diuresis produced by mercurhydrin when BAL was also given. Ginzler (58) has studied the anatomical lesions caused by mercury, and has shown that appropriate therapy with BAL can completely prevent the necrotizing action of mercuric chloride on the renal tubules.

**Gold.** The antidotal effect of BAL and of glutathione against the toxic actions of gold chloride and myocrisin (sodium aurothiomalate) on the brain pyruvate-oxidase system were studied by Thompson and Whittaker (180). Their results showed that under these *in vitro* conditions BAL effectively detoxicates both these compounds; some protection was also afforded by glutathione, although it was much less effective than BAL.

Experiments with rats have shown that an increase in survival time after the administration of gold sodium thiosulphate or gold thioglucose can be obtained by treatment with BAL. This effect was not obtained when the gold was given as sodium aurothiomalate; if, however, sub-lethal doses of this compound were given to young rats, the administration of BAL caused an increased growth rate as compared with that of control animals. This evidence indicating a therapeutic action of BAL in experimental gold poisoning is however offset by the finding that the association of BAL and gold produces a nephrotoxic action (90). With regard to this latter finding it must be realized that the doses, both of gold and of BAL, used in experimental work in animals are very much greater than those employed therapeutically in man.

A number of short papers have also appeared dealing with the value of BAL in the treatment of toxic reactions arising in the course of gold therapy of rheumatoid arthritis (102, 23, 130, 92, 154, 148). Cases of exfoliative dermatitis, thrombocytopenic purpura and granulocytopenia have been described, and, in general, it has been concluded that treatment with BAL has been of value, in some instances striking responses being obtained. Ragan and Boots (130) have stated that a significant increase in the urinary excretion of gold occurs after BAL therapy; they also reported an exacerbation in the symptoms of the rheumatoid arthritis following this increased elimination of gold.

**Lead.** The possibility of treating lead poisoning with dithiols has been investigated by several groups of workers, but the results which have been obtained are much less clear-cut than with the other metals which have been considered above. Braun *et al.* (11) first reported that the mortality of rabbits poisoned intraperitoneally with lead nitrate was increased above that of control animals by treatment with BAL. Weatherall (192), working with mice, has claimed, however, that the mortality caused by repeated intraperitoneal injections of lead acetate can be slightly reduced by BAL and more significantly by BAL glucoside. *In vitro* he found that both these compounds prevented the decrease in the fragility of washed erythrocytes brought about by lead acetate, while in rabbits each significantly decreased the anemia caused by a single dose of lead acetate given by mouth; the mortality rate however was not significantly affected, while the coproporphyrinuria was actually increased. Weatherall concludes that the action of BAL in lead poisoning is restricted to an inactivation of lead ions not yet taken up by cells, and that there is no evidence of

'de-leading' and therefore no reversal of poisoning comparable to that which occurs with arsenicals.

Germuth and Eagle (53) have also reported a failure to protect rabbits poisoned with intravenous or subcutaneous injections of lead acetate.

On the other hand, injection of BAL increases the urinary excretion of lead, and markedly affects the distribution of the metal in the tissues (57). It has also been claimed that the nephrotoxic action of lead in rats can be prevented by the intraperitoneal injections of an aqueous solution of BAL (22).

Turning to the effects of lead in man Ryder, Cholak and Kehoe (140) reported that treatment with BAL of a group of men who had been exposed to an industrial hazard of lead brought about a rapid fall in the concentration of lead in the whole blood, although causing no change in the plasma level. It also caused a considerable increase in the urinary excretion of lead, but despite these findings it was concluded that BAL therapy is of little value in the treatment of lead poisoning.

Telfer (173) has reported a case of chronic lead poisoning which he treated with BAL. An increased excretion of lead occurred during two courses of therapy; the conclusion was reached that BAL was deserving of a further trial both as a diagnostic test (increased excretion of lead) and possibly as a therapeutic aid.

**Cadmium.** Some careful and detailed experimental work has also been carried out with this metal, since although cadmium poisoning is not frequently encountered industrial accidents with it have occurred. The biochemical aspects of its mode of action have been studied by Simon, Potts and Gerard (147), who investigated the action of cadmium ions on tissue respiration and on a number of partially purified enzymes. They found succinic dehydrogenase to be most readily inhibited by cadmium among the systems studied by them; choline oxidase was also inhibited but to a smaller extent, while alkaline phosphatase, mono-amine oxidase and cytochrome oxidase were only slightly affected, if at all.

These workers also used the succinic dehydrogenase from lung tissue for an *in vitro* study of the ability of thiols to reverse the inhibition produced by cadmium chloride. They observed an increasing efficacy with the number of SH groups in the molecule, a trithiol being the most active agent, dithiols of intermediate and monothiols of least value. Barron and Kalnitsky (5) also investigated a large number of dithiols as antidotes against the inhibition of muscle succinic oxidase by bismuth, mercury, cadmium, vanadium, zinc, lead and selenium. They concluded that two 1:3- substituted dithiols, 1:3-dimercaptopropanol and 1:3-dimercaptopropane, were distinctly better than BAL not only for the reversal of cadmium inhibition but also for that caused by the other metals.

In the treatment of cadmium poisoning in animals apparently conflicting results have been obtained. In mice the mortality was reduced from 93 per cent to 7 per cent when intramuscular BAL was given after intraperitoneal  $\text{CdCl}_2$ , and in dogs from 100 per cent to 42 per cent when the  $\text{CdCl}_2$  was inhaled as a mist (182). Similar results were obtained in dogs with cadmium aerosols by Harrison *et al.* (66), but on the other hand prophylactic BAL appeared to have a deleterious effect (182). It appears that BAL does not affect in any characteristic way the distribution of cadmium in the tissues (66) or the concentration or rate of removal of cadmium from

the lungs, while when BAL is administered before the cadmium it actually fixes more of the metal in the lungs (182).

It has also been shown that although BAL appears to exert a therapeutic effect against cadmium, renal insufficiency may develop in the treated animals (182, 55). It is concluded that this is brought about as a result of the tubular reabsorption causing a concentration of the cadmium mercaptide in the epithelium of the renal tubules, with a subsequent release by intracellular oxidation of toxic amounts of cadmium ions.

Death can therefore result from renal insufficiency in spite of the fact that BAL has probably removed cadmium from essential tissue enzymes elsewhere. Therapy with BAL-glucoside appears to be more successful, and the incidence and degree of renal damage in surviving animals is distinctly less than that observed after therapy with BAL by Gilman *et al.* (55). These workers also found the monothiols, 1-thio-sorbitol and 1-thioxylitol of some value therapeutically in cadmium poisoning. According to Teppermann (174) when BAL is used as the antidote the increased excretion of cadmium occurs in both urine and feces, while after treatment with BAL-glucoside it occurs chiefly in the urine. This finding supports the view that the cadmium complex formed with the BAL-glucoside remains extracellular, and is not re-absorbed to the same extent as the BAL compound. It seems possible therefore that a combination of the two drugs might produce a considerable increase in therapeutic efficiency, since the BAL should remove cadmium from the cells, and with the subsequent establishment of an equilibrium between the BAL and the BAL-glucoside the accumulation of toxic amounts of cadmium in the kidney might be avoided.

*Antimony.* *In vitro* evidence of antidotal effects of BAL against the toxic action of antimonials has been obtained by Thompson and Whittaker (180), who showed that BAL, when present in a molar ratio of 6:1, was able to bring about a significant degree of reversal of the toxic action of tartar emetic on the brain pyruvate oxidase system, even when the poisoning had been allowed to proceed for 20 minutes. It was also shown to be able to protect the enzyme from inhibition by stibophen (Fuadin), a complex compound of trivalent antimony and sodium catechol disulphonate. In the case of the muscle succinic-oxidase system Barron and Kalnitsky (5) found that the amount of BAL required to reverse the action of Fuadin was too large to be practicable.

In animal experiments Gammill, Southam and van Dyke (51), using rats, obtained an increased mortality rate when BAL was used as an antidote to the intraperitoneal injection of Fuadin, Neostam (glucoside of sodium *p*-aminophenylstibonate) or Neostibosan (diethylamine *p*-aminostibonate), although it did prove of some value against tartar emetic. In rabbits, however, Eagle and associates (38) obtained successful protection against intravenous Anthiomaline (antimony lithium thiomalate), Fuadin, tartar emetic and *p*-methylphenol stibonic acid, confirming the results of Braun *et al.* (11), reported earlier, who used tartar emetic, Fuadin and Neostam.

*Other Metals.* Indications that treatment with dithiols may be of value in yet other forms of metallic poisoning are provided by further experiments of Braun *et*



*al.* (11), who found that the LD<sub>50</sub> doses of nickel chloride, potassium bismuthotartarate and potassium chromate were significantly increased by intramuscular injections of BAL shortly after the administration of the metal salt. Olcott and Riker (115), however, have reported that BAL is of no value in experimentally-produced argyria in rats. Amdur (2) treated 3 cases of accidental exposure to tellurium fumes with BAL, and suggested from the short duration of the symptoms that a beneficial result had been obtained.

Some interesting observations have been reported by McCance and Widdowson (106) on the effect of BAL-glucoside on the heavy metals normally present in the serum in man, since it was thought possible that BAL might also form soluble compounds with these metals, and so cause an increase in their excretion in the urine. Urine was therefore collected from 6 subjects before and after the intravenous injection of BAL-glucoside. Analysis of the urine specimens showed that the drug increased the urinary excretion of copper about 20 times and that of zinc about 5 times; the excretion of iron however did not appear to be significantly affected. In view of the prevalence in certain areas of diseases of sheep associated with either a deficiency or an excessive storage of copper a similar investigation has been carried out with sheep (108); in this species the rate of excretion of copper was increased approximately 30 times by an injection of BAL-glucoside. Stocken and Thompson (163) have further shown that BAL itself causes a similar increase in copper excretion in the rat, while in man a single injection of 3 mg. BAL/kg. body weight produces a marked elevation of the copper excreted by the kidneys in the succeeding 2 hours (100). Stewart and Robertson (160) administered a prolonged course of BAL injections to a sheep, and obtained an estimated loss of 7 mg. copper over a period of 60 days.

Macnider (99) has reported that BAL, given twice daily for 8 days, is unable to prevent toxic signs developing in the livers of dogs poisoned with uranium nitrate.

#### MISCELLANEOUS APPLICATIONS OF BAL

Although this review is primarily concerned with the reactions of thiols with metals, a brief reference will be made to some miscellaneous applications of BAL although in some instances the relevance to metals, or indeed the mechanism underlying the observations, is far from clear.

Chesler and Tislow (21) have reported that BAL, given either orally or parenterally, protects rats against the diabetogenic and lethal effects of subsequent injection of alloxan, a reaction which is to be anticipated on chemical grounds. Similar results were obtained by Lazarow (89).

In view of the suggestion of Du Bois (33) that thiol-containing compounds are inhibited by  $\alpha$ -naphthylthiourea, BAL and 1-thiosorbitol have been studied as possible antidotes for phenylthiourea poisoning (70). It was found that thiosorbitol affords considerable protection if given immediately, whereas BAL increases rather than diminishes the toxicity.

In the field of antibiotics Anderson and Chin (3) have reported that BAL at a dilution of 1:100,000 is synergistic with subtilin in bacteriostatic action on all the strains of mycobacteria tested, but not on *M. lysodeikticus*. The action of strepto-

mycin is potentiated against pathogenic mycobacteria, although against *M. lysodeikticus* it is inactivated in the presence of BAL. It is of interest to note that Johnson, Anken, Scudi and Goldberg (79) have reported that bacitracin is also inactivated by BAL. Chain, Philpot and Callow (17) have used BAL to prevent the catalytic effect of metal ions on the alcoholysis of the penicillins.

Since one theory of tumor formation has suggested that the carcinogen reacts with free SH groups in the cell (25), Lusky, Braun and Woodward (97) have investigated the possibility that the provision of a competing thiol might prevent tumor development. Using comparable groups of 55 mice they were able to show that 17 weeks after the application of benzpyrene, 82 per cent of the control mice had developed skin tumors as compared with 58 per cent in the group anointed with BAL in addition to benzpyrene, a difference which was shown to be statistically significant.

In a recent investigation of the succinic oxidase system Slater (153) has reported that although the complete system was practically completely inhibited by BAL under his conditions, neither the cytochrome oxidase nor the succinic dehydrogenase is significantly affected. These findings have led him to the conclusion that BAL inactivates a hitherto unknown component of the complete system, this labile factor being concerned in the transfer of electrons from cytochrome *b* to cytochrome *c*.

In a study of the toxic principles of argemone oil, Sarkar (142) has isolated dihydrosanguinarine and sanguinarine, and has shown that the latter inhibits pyruvate oxidation by brain preparations. He has also obtained successful *in vitro* protection of this enzyme system from inhibition by sanguinarine, and has protected rats from the effects of an intraperitoneal injection of a lethal dose of the poison.

Dustin (35) has reported that although BAL is capable of reversing to a certain extent the mitotic poisoning produced by sodium arsenite, it is itself a mitotic poison, with an activity similar in type to that of colchicine.

#### BAL-INTRAV

The preparation by Danielli *et al.* (28) of the glucoside of BAL (BAL-Intrav) has already been mentioned. This compound is very markedly less toxic than BAL, the LD<sub>50</sub> for rabbits being greater than 5 g/kg., i.e., less than one hundredth the toxicity of BAL. It represents an advance of considerable theoretical importance, and in view of its water-solubility it was hoped that it might also constitute a practical advance in providing a non-toxic dithiol suitable for intravenous use. Some difficulty has, however, been experienced in the preparation of this compound, or of related compounds, in a pure and stable form, but work in this connection is still progressing. Mention has already been made of the therapeutic applications of this glucoside.

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## METABOLISM OF PARASITIC HELMINTHS<sup>1</sup>

ERNEST BUEDING

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IN HIS BOOK, *An Introduction to Comparative Biochemistry*, Baldwin (13) has commented on the disproportionality between our knowledge concerning the biochemistry of vertebrates and bacteria on one hand and that of invertebrate metazoa on the other. This author has pointed out that biochemistry is "in danger of growing without properly differentiating, of developing members adult in size while possessing only the most primitive supporting structures and correlating apparatus," and that "only by ensuring the proportionate growth of all its members can the full humanitarian value of biochemistry be realized."

As with other invertebrate metazoa, information about the metabolism of parasitic helminths is very limited. On the other hand, the distribution of parasitic worms is extremely wide. At least 800 million human beings are infested with helminths and in many of them multiple infestations occur; Stoll (176) has estimated that the total number of helminthiases exceeds two billion. The metabolism of parasitic worms, as a group, is of particular interest also, because of the great differences in the chemical composition of the habitats of various species. In view of these marked environmental differences which include also differences in the oxygen tension, it is not surprising that the metabolism of these organisms varies from one species to another to a much greater extent than does that of the vertebrates.

Several subjects discussed in this article have been reviewed previously by MacCoy (125), Von Brand (199, 210), Von Brand and Jahn (204), Lapage (113), Smyth (170) and Hobson (93).

<sup>1</sup>The investigations of the author quoted in this review were carried out with the generous support of a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.



## PROBLEM OF OXIDATIVE METABOLISM

Since many parasitic worms live in the intestinal tract where the oxygen tension is low it has often been assumed that the metabolic activities of these organisms in their physiological habitat are primarily, if not exclusively, anaerobic. Indeed, it has been postulated by some investigators (227, 161, 111, 233) that oxidative reactions are not essential for their survival. Others (59, 163, 164, 127, 46, 114, 187) have categorically rejected such a possibility. The presently available evidence does not permit this controversial question to be answered by a generalization.

Parasitic helminths cannot be considered as obligatory anaerobic organisms, since they will take up oxygen if it is available to them (171, 52, 5, 2, 81, 88, 89, 195, 110, 26, 23, 25, 203, 205, 211, 150) and since they will survive in environments whose oxygen tension is high. However, many of these organisms have access only to a small amount of oxygen, because their habitats, such as the intestinal tract or the bile ducts, contain very little of this gas (180, 193, 195, 182, 22, 121). An exception may be found in the case of the intestinal parasites of the pig, because the oxygen tension in the gastrointestinal tract of this animal frequently is considerably higher than in that of other mammals (193, 195). Since the rate of respiration of several intestinal helminths varies directly with the oxygen tension of the medium (81, 87, 109), respiration of these organisms must be extremely small at the low oxygen tensions usually prevailing in the intestinal tract. Furthermore, large intestinal helminths such as *Ascaris* and certain tapeworms can survive under anaerobic conditions for relatively long periods of time (227, 90, 233, 31, 19, 183, 171, 52, 177, 237, 31). Only when frequent convulsions of *Ascaris* are induced by electrical shocks do these parasites die more rapidly in an oxygen-free atmosphere than in air (163). Therefore, aerobic metabolism appears to supply the additional energy required by heightened muscular activity. However, under physiological conditions such activity must occur but rarely, if ever, since, according to radiological evidence, the motility of *Ascaris* in the human intestinal tract is very low (115). Consequently, it appears unlikely that the large helminths which inhabit the intestinal tract and the bile ducts derive a major portion of their energy requirements from aerobic reactions. On the other hand, it cannot be stated that the small amount of respiratory metabolism presumably occurring at the low oxygen tensions of their habitat is of no physiological importance. More precise information about the significance of their respiratory metabolism could be obtained by the development of nutritionally adequate media for culturing these parasitic worms aseptically *in vitro*. This would make it possible to investigate their response to various oxygen tensions and to anaerobiosis over prolonged periods of time.

Special conditions under which oxygen is supplied to intestinal helminths exist in the host-parasite relationships of two types of nematodes: 1) Wells (235) has shown that the hookworm, *Ancylostoma caninum*, obtains oxygen from oxyhemoglobin by sucking the blood of the host; the amount of blood withdrawn by these nematodes is larger than is commonly appreciated, and at least in part, may account for the anemia observed in this parasitic infestation (64). 2) If the intestinal parasite is of sufficiently small size diffusion of oxygen into the organism may occur even at low oxygen tensions. This appears to be the case with the nematodes,

*Ostertagia circumcincta* and *Trichostrongylus spiralis*, which live in the digestive stomach (abomasum) of sheep (45, 46). As pointed out by Von Brand (199), these organisms are about 30,000 times smaller than *Ascaris* and therefore, the ratio of surface area to volume of such nematodes favors a high rate of respiration, even at low oxygen tensions. The available evidence indicates that respiratory metabolism is essential for these two types of intestinal parasites which have access to large amounts of oxygen: Davey (45, 46) has demonstrated that certain small nematodes, *Ostertagia circumcincta* and *Trichostrongylus spiralis*, are extremely sensitive to lack of oxygen and that they survive for periods five to fifteen times as long in an atmosphere of air as under anaerobic conditions. In addition, according to the findings of Von Brand and Otto (198), the carbohydrate metabolism of hookworms resembles that of non parasitic worms which depend on aerobic metabolism to a much greater degree than *Ascaris*. Recent observations of Rogers (152) indicate that aerobic mechanisms also play an important role in the metabolism of other small intestinal nematodes (*Nippostrongylus muris*, *Nematodirus* spp. and *Hoemonchus contortus*).

The habitats of tissue helminths usually have a higher oxygen tension than those of intestinal worms. However, a relatively high oxygen tension of their environment does not necessarily indicate that such organisms are greatly dependent on respiratory metabolism. This is illustrated by the differences in the behavior of two tissue helminths, the filarial nematode, *Litomosoides carinii*, and the trematode, *Schistosoma mansoni*. The former lives in the pleural cavity, the latter in the blood plasma of the mesenteric and portal veins. The oxygen tension of the pleural cavity varies between 12 and 25 mm. Hg (74, 224, 44, 75, 178), that of the portal vein between 49 and 66 mm. Hg (17, 51, 92). Through the use of the cyanine dyes (21, 234, 239, 138), it has been possible to investigate to what degree respiratory metabolism is essential for these two parasites. The cyanines in extremely low concentrations inhibit the oxygen uptake of the filariae (234, 26, 138) and of the schistosomes (25). This effect of the cyanines is not restricted to conditions *in vitro*; if the dyes are administered to the mammalian hosts, the respiration of the parasites is markedly inhibited (24, 26). Reduction of their respiratory metabolism results in the death of the filariae (234, 26), but the schistosomes survive (24, 25) and continue to produce eggs (139) under these conditions despite the fact that the oxygen tension of their habitat is higher than that of the filariae. Furthermore, anaerobiosis or inhibition of respiration produced by the cyanine dyes results in a compensatory increase in the rate of glycolysis of the filariae (26), i.e., a much larger proportion of the carbohydrate utilized by the organism is converted to lactic acid. With *S. mansoni*, the production of lactic acid from glucose is the same in the presence or absence of oxygen, or of cyanine dyes (25). The possibility cannot be excluded that *S. mansoni* may require a small proportion of its normal oxidative metabolism. Although this organism survives when 80 per cent of its respiration is inhibited by a cyanine dye, death might occur if respiration were to be inhibited completely. In this eventuality, the difference, in their respective dependence on oxidative metabolism, between *L. carinii* and *S. mansoni* would be a quantitative rather than a qualitative one, and the oxygen requirement of *S. mansoni* would be comparable to that of another tissue parasite, the larval nematode of *Eustrongylides ignotus*. Von Brand and Simpson (208) found that this organism survives at relatively low oxygen tensions in nutritionally ade-

quate media for many months, but dies rapidly under strictly anaerobic conditions. Another tissue nematode, the larval form of *Trichinella spiralis*, appears to require oxidative metabolism for the preservation of its motility, but not for its survival (171).

Harnisch (80, 83, 85, 87, 82), Friedheim and Baer (67), and Von Brand (211) have demonstrated a direct proportionality between the oxygen tension of the environment and the rate of respiration of parasitic worms. This is observed also when minced tissue, instead of the whole organism, is used (80, 82), indicating that a more rapid diffusion of oxygen at higher tensions does not account for this phenomenon. On the other hand, the oxygen uptake of tissues from predominantly aerobic organisms, such as vertebrates, insects, crustaceans and many free-living worms, is much less dependent on the oxygen tension, their respiration remaining constant above a certain minimum level of the oxygen tension (84, 86, 80, 82, 122, 96, 38a). On the basis of these observations, Harnisch (83, 85, 87, 82) has postulated that any organism whose tissue respiration, under optimal conditions of diffusion, is dependent to a large degree on the oxygen tension, has a predominantly anaerobic type of metabolism in its physiological habitat. Should this hypothesis prove to be correct, a simple experimental procedure would be available to determine whether the metabolism of a given organism is primarily aerobic or not. However, according to observations of Van Grembergen (187, 189) respiration of the tissue pulp of *Moniezia benedini* and of *Fasciola hepatica*, two helminths possessing a predominantly anaerobic metabolism, is independent of the oxygen tension over a wide range. Furthermore, the respiration of the filarial worm *L. carinii*, which has a predominantly aerobic type of metabolism, is at least as dependent on the oxygen tension as the respiration of *S. mansoni*, whose metabolism is primarily anaerobic (27). Consequently, the relationship between the oxygen uptake of a parasitic helminth and the oxygen tension cannot be considered as a reliable criterion for the relative importance to the parasite of anaerobic and aerobic metabolism (212).

The evidence discussed in the foregoing paragraphs supports the conclusion of Von Brand (199, 210) that the dependence on respiratory metabolism varies greatly from one parasitic helminth to another and that it must be investigated for each individual species. With some of them, the requirement for oxidative reactions is high and short periods of anaerobiosis or partial inhibition of their oxygen uptake results in their death. Others can survive when the oxygen tension of their habitat is extremely low, or when their respiration is almost completely inhibited. For some of the latter, respiratory metabolism may not be essential at all and may be merely a rudimentary function carried over from a previous stage of the life cycle of the parasite, or from a different, but phylogenetically related species.

#### CARBOHYDRATE METABOLISM

With the exception of *Ancylostoma caninum* (198), of microfilariae of *Wuchereria bancrofti* (20) and of adult *Trichinella spiralis* (132), parasitic helminths in general are characterized by a high glycogen content. In many respects the glycogen isolated from *Ascaris lumbricoides* is similar to that from mammalian tissues (62, 226, 9). The same appears to be true for the polysaccharide present in *Moniezia expansa* (131), *Fasciola hepatica* (131), *Cysticercus fasciolaris* (153), *Macracanthorhynchus hirudinaceum* (197, 202) and *Lilomosoides carinii* (26). Utilization of glycogen is an important

mechanism by which parasitic worms obtain energy. Absence of carbohydrate from their environment results in a pronounced decrease in the glycogen content of the parasites. This was discovered in *Ascaris lumbricoides* by Weinland (227) and subsequently has been confirmed for this organism (195, 111, 213, 196, 161) as well as for all other parasitic helminths whose glycogen metabolism has been investigated (223, 183, 200, 26, 194, 142, 143, 144, 124). Incubation of extracts of *Ascaridia galli* and of *Nematodirus spp.* results in a decrease in endogenous glycogen (151). Utilization of endogenous glycogen during starvation of parasitic worms has been demonstrated not only *in vitro* but also within the host. Reid (142-144) has observed that the glycogen contents of two intestinal helminths of the fowl, the cestode *Rallietina cesticiillus* and the nematode *Ascaridia galli*, are reduced to about 10 per cent of their original values after starving the host for 20 to 24 hours. On the other hand, the starvation or the feeding of large amounts of glucose to dogs infested with *Ancylostoma caninum* had no effect on the glycogen stores of these hookworms (198). In this particular case the parasites obtain their food by blood-sucking (235) and are thus independent of nutrients supplied by the intestinal contents.

Less quantitative data are available on the metabolism of simple sugars by parasitic worms. Weinland (227) has reported that the glucose content of *Ascaris lumbricoides* decreases during starvation. However, Foster (65) and Von Brand (195) did not detect any significant amounts of reducing sugars in *Ascaris*. According to Von Brand (195, 210), the high glucose values preceding starvation reported by Weinland (227) were artefacts produced by hydrolysis of glycogen during the determination of free reducing sugar. Glucose, fructose, and, to a lesser extent, galactose and maltose, increase the period of survival of *Fasciola hepatica* in a buffered salt solution, but lactose and sucrose were ineffective (172). Survival *in vitro* of the larval *Eustrongylides* is prolonged by glucose, but is not affected by fructose, mannose or maltose (206). When added to the medium or when injected, glucose, and to a lesser degree, fructose or galactose, have a glycogen-sparing effect on *Ascaris lumbricoides* (229). An increase in the glycogen content of *Ascaris lumbricoides* by the injection of glucose was not observed consistently (229). It is possible that glycogen synthesis from glucose and other substrates could be demonstrated more readily if the glycogen stores of this organism were depleted by starvation. A glycogen-sparing effect of glucose was observed also in *Moniezia expansa* (194) and the larval *Eustrongylides* (208). Addition of glucose to the medium resulted in a slight increase in the glycogen content of *Moniezia expansa* (222). This effect was not observed with fructose, maltose, galactose, arabinose, glycine or mucin (222). Markov (124) has reported a very pronounced decrease in the glycogen content of the plerocercoids of the cestodes *Diphyllbothrium latum*, *Ligula intestinalis*, *Eubothrium rugosum* and *Tri-*ae*nophorus nodulosus* during incubation in Ringer-Locke solution. Addition of glucose to this medium produced a slight increase in the glycogen content of the parasites. A very marked rise in the glycogen stores of the filarial worm *Litomodoides carinii* was observed when this organism was incubated aerobically in a glucose-containing medium (26). Polysaccharide synthesis by extracts of *Ascaridia galli* in the presence of adenylic acid and glucose-1-phosphate has been demonstrated by Rogers (151). Such an observation suggests that glycogen synthesis in this organism involves enzymatic mechanisms similar to those discovered by Cori and his coworkers (39-42,

6g) in mammalian tissues. The tissue parasites, *L. carinii* and *Schistosoma mansoni*, have a very high rate of glucose utilization. The Schistosomes can remove, from their surrounding medium, in one hour an amount of glucose equivalent to almost one fifth of their dry weight (23, 25). The rate of glucose utilization by *Lilomosoides carinii* is about half as great as that of *Schistosoma mansoni* (26). These two helminths appear to have the ability to oxidize hexoses since the addition of glucose or fructose produces an increase in their respiration (26, 27). Similarly, the oxygen uptake of *Diphyllobothrium latum* was greatly increased by glucose (67). On the other hand, glucose did not increase the respiration of *Moniezia benedini* (187) or of larval *Trichinella spiralis* (171). Glucose utilization by *Schistosoma mansoni* was

TABLE 1. PRODUCTS OF THE CARBOHYDRATE METABOLISM OF PARASITIC HELMINTHS

SPECIES	PRODUCTS
<i>Ascaris lumbricoidea</i>	n-valeric acid acetic acid lactic acid formic acid butyric acid (?) propionic acid (?) aldehydes (?) ethyl alcohol (?)
<i>Parascaris equorum</i>	valeric acid lactic acid
<i>Moniezia expansa</i>	higher fatty acids lactic acid succinic acid
<i>Fasciola hepatica</i>	higher fatty acids volatile fatty acids
Larval <i>Trichinella spiralis</i>	CO <sub>2</sub>
<i>Schistosoma mansoni</i>	(D, L)-lactic acid
<i>Lilomosoides carinii</i>	L (+)-lactic acid acetic acid
<i>Dracunculus insignis</i>	lactic acid

found to be independent of the glucose concentration in the medium. On the other hand, the rate of fructose utilization by this organism was directly proportional to the concentration of this sugar (27).

The observations summarized above suggest that several hexoses, notably glucose, can be converted to glycogen by parasitic helminths and that they can replace this polysaccharide as a fuel. Under physiological conditions the habitats of parasitic worms contain an adequate supply of glucose for these reactions.

Lower volatile and higher fatty acids are the main products of the anaerobic carbohydrate metabolism of intestinal helminths. Weinland (227, 231) isolated the acids produced by *Ascaris lumbricoidea* as their calcium salts and concluded that this parasite converts glycogen predominantly to valeric acid and to a smaller extent to caproic acid. This was confirmed by Flury (62). According to this author, *Ascaris lumbricoidea* also produces butyric acid, propionic and small amounts of formic acid. In addition, several colorimetric tests suggested the presence of aldehydes in media

in which these worms had been incubated (62). Von Brand (195) found that this helminth produces small, but measurable amounts of lactic acid. Waechter (214) isolated, from media in which *Ascaris lumbricoides* had been incubated, an acid whose copper salt was obtained in crystalline form. The shape of these crystals was identical with that of copper-n-valerate but showed no resemblance to the copper salt of iso-valeric acid. The preparation and identification of the phenyl-phenyl-acyl derivative of n-valeric acid from similar media by Oesterlin (130) has established that this acid is a major product of the carbohydrate metabolism of *Ascaris lumbricoides*. It is interesting to note that valeric acid is also a product of the metabolism of the free-living worm, *Lumbricus terrestris* (117). In addition to n-valeric acid Oesterlin (130) demonstrated the production of acetic acid by *Ascaris*. While formation of butyric acid could not be established conclusively, strong evidence for the production of formic acid was obtained by this author (130). He found no evidence that *Ascaris* produces iso-valeric acid. Circumstantial evidence that ethyl alcohol is a product of the carbohydrate metabolism of *Ascaris* has been reported (62, 105).

According to Toryu (184), the major product of the carbohydrate metabolism of *Parascaris equorum* is valeric acid and only a small fraction of the acids formed by this organism can be accounted for by lactic acid. These observations confirmed the earlier work of Fischer (59). Von Brand (194) found several differences between the metabolic products of *Ascaris lumbricoides* and those of *Moniezia expansa*. The latter organism produces no volatile acids, but large quantities of higher fatty acids, some of which appear to be monohydroxy acids (194). Furthermore, Von Brand (194) observed that *Moniezia* forms succinic acid and greater amounts of lactic acid than *Ascaris*. Lactic acid production accounted for 16 per cent of the glycogen utilized by *Moniezia expansa*. Weinland and Von Brand (223) and Stephenson (173) have found that higher fatty acids are also the major products of the carbohydrate metabolism of *Fasciola hepatica*. This trematode, unlike *Moniezia*, produces small amounts of volatile fatty acids.

In addition to the above-mentioned products, it has been reported frequently that parasitic helminths form carbon dioxide from carbohydrate. CO<sub>2</sub> production may be due either to the actual production of this gas or to the formation of an acid or acids which liberate CO<sub>2</sub> from bicarbonate present in the medium or preformed in the helminth. Furthermore, CO<sub>2</sub> liberated by either of these mechanisms may be partially bound and retained by the proteins or other buffers of the organism (219). Unless corrections are made for these two sources of error, a quantitative evaluation of CO<sub>2</sub> production is not possible.

Slater (163, 164) has stated that fatty acids present in solutions in which parasitic helminths had been kept were not produced by these organisms, but by bacterial contamination. The evidence available does not support such an hypothesis: 1) Although *Ascaris lumbricoides* and *Moniezia expansa* live in the same habitat, and, presumably, carry the same bacterial contaminants, different metabolic products are formed by these two parasites. 2) Higher fatty acids not only are present in the medium in which *Fasciola hepatica* has been kept, but they also have been demonstrated by histochemical methods within the excretory system of this organism (192, 223), and, in *Ascaris*, the same lower fatty acids which accumulate in the medium can be isolated from the body of the worm itself (62, 157). 3) In many experiments

the worms were kept in a salt solution containing no glucose or other substrate. It appears quite improbable that significant fatty acid production by bacteria could occur in such non-nutrient media. Even when glycogen or glucose had been added to such media, no increase in valeric acid content occurred after removal of *Ascaris* (231). 4) Slater (163) quotes experiments of Fischer (59) which indicate that, in contrast to intact *Parascaris equorum*, the only acid formed by extracts from this animal is lactic acid. However, in these experiments, the total acidity of the extracts was determined by boiling them before titration. Thus, volatile acids originally present in the extracts could have been driven off by such a procedure. Furthermore, Fischer could detect no significant lactic acid formation when his extracts were incubated for 15 hours at 37° C. Only after this period did a rapid increase in lactic acid occur. Such an observation suggests that the production of lactic acid was due to bacteria (119) and not to the activity of the tissue extract itself, since the enzymatic activity of the latter presumably decreases rather than increases with time. 5) Weinland (228) has shown that volatile fatty acids were formed by extracts of *Ascaris lumbricoides* to which antiseptics had been added. Sterility tests revealed the absence of bacteria in these extracts (228). 6) It has been pointed out above that the rate of acid production, associated with the growth of contaminating bacteria, should increase with time. As shown by Toryu (184) the formation of volatile acids during incubation of *Parascaris equorum* remains constant over prolonged periods of time.

A type of metabolism, as yet unexplored, which differs from that of other parasitic helminths, is found in *Trichinella spiralis*. Stannard, McCoy and Latchford (171) have observed that these organisms produce a considerable amount of carbon dioxide, but no organic acids whatsoever. While the adult and larval forms of *Trichinella* are alike in their lack of production of acids, the adult trichina contains, in contrast to the larvae, little or no glycogen (132). Investigation of the carbohydrate metabolism of larval and adult trichinellae should prove of great interest and might reveal some hitherto unknown metabolic reactions.

Until recently it appeared that, in contrast to tissues of vertebrates and to many bacteria, the major portion of the carbohydrate utilized by parasitic helminths was, in no instance, converted to lactic acid. However, in the case of the tissue helminths, *Lilomosoides carinii* and *Schistosoma mansoni*, lactic acid production accounts for at least 80 per cent of the total carbohydrate utilized anaerobically by these two organisms. This was established by isolation of the acid from media in which these organisms had been incubated and by subsequent preparation and identification of the benzimidazole and the p-bromophenacyl-derivatives (26, 27). The filariae form only L (+)-lactic acid (26) while the schistosomes produce racemic lactic acid (27). Only a small fraction of the anaerobically consumed carbohydrate is converted to acetic acid by *Lilomosoides carinii* (26). Evidence also has been obtained that lactic acid is the major end product of a third tissue helminth, the nematode, *Dracunculus insignis* (28), which lives in the subcutaneous tissues of raccoons (33).

Pasteur (134) discovered that the rate of fermentation by yeast is considerably higher under anaerobic conditions than in the presence of air. Subsequent investigations have revealed that oxygen decreases the utilization of carbohydrate and suppresses or decreases the accumulation of the products of anaerobic metabolism in a large variety of animal tissues and bacteria (50). Twelve times more energy is

liberated from the oxidation of one mole of glucose to  $\text{CO}_2$  and water than from its fermentation (11). Consequently, in organisms which possess mechanisms for the complete oxidation of carbohydrate and which are able to utilize the energy derived from this process, fermentations and other anaerobic reactions may be reduced greatly by the presence of oxygen. Thus, in most vertebrate tissues, anaerobic glycolysis<sup>2</sup> is much higher than aerobic glycolysis (218). In the free living worm, *Lumbricus terrestris*, glycogen consumption is six times lower under aerobic than under anaerobic conditions (118) indicating a high efficiency in the oxidative utilization of carbohydrate. That the earthworm has a primarily aerobic type of metabolism is revealed by the fact that it does not survive in an oxygen-free atmosphere for a period of more than 24 hours (118).

A survey of parasitic helminths, in which aerobic and anaerobic fermentations have been measured, reveals that the presence or absence of oxygen has little or no effect on fermentations in those organisms which have a predominantly anaerobic type of metabolism. Conversely, in worms whose dependence on respiratory metabolism has been established, a marked reduction of fermentation occurs in the presence of oxygen. The available evidence indicates that the large intestinal worms and the tissue helminth *Schistosoma mansoni* have a predominantly anaerobic type of metabolism (see above). In such organisms oxidative metabolism has little or no effect on their rates of fermentation. Alt and Tischer (5) and Von Brand (194) found no difference between the total amounts of acids formed by *Moniezia expansa* under aerobic and anaerobic conditions. Therefore, oxygen appears to have no inhibitory effect on the rate of fermentation of this organism, provided the acids formed anaerobically are the same as those produced aerobically. In *Ascaris lumbricoides* anaerobic production of valeric acid is twice as high as in air and anaerobic glycogen consumption is 1.2 to 1.3 times higher than that occurring aerobically (195). Evidently the presence of oxygen results in a significant, but relatively slight reduction of fermentation of this parasite. In *Parascaris equorum* this effect is still smaller. According to Toryu (183, 184), the production of valeric and lactic acids and the utilization of glycogen by this helminth are only 3 to 10 per cent lower in air than in nitrogen. Carbohydrate utilization and lactic acid production by *Schistosoma mansoni* are the same under anaerobic conditions and in atmospheres whose oxygen content varies between 1 and 100 per cent (25, 27).

On the other hand, pronounced differences between the rates of aerobic and anaerobic fermentation have been observed in two parasitic helminths whose dependence on oxidative metabolism has been established. Von Brand and Simpson (197, 208) have demonstrated that the larval nematode *Eustrongylides*, when kept anaerobically, utilized three times more glycogen than under aerobic conditions. Anaerobically, this parasite produces a considerable amount of acid but this does not occur in the presence of air (197, 203). Anaerobically, the filarial worm, *Litomosoides carinii* converts 80 per cent of the utilized carbohydrate to lactic acid, but under aerobic conditions only 30 to 45 per cent is converted (26). Inhibition of respiration

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<sup>2</sup>The term 'fermentation' is used for anaerobic metabolism of carbohydrate while 'glycolysis' is reserved for the production of lactic acid from carbohydrate, a special case of fermentation (see also Von Brand, 210).



by cyanide or cyanine dyes results in a compensatory increase in the rate of glycolysis of the filariae (26). Acetate production by this organism is 2.5 to 3 times higher aerobically than anaerobically (26).

The observations discussed above lead to the following tentative conclusion: The more a parasitic helminth is dependent upon anaerobic metabolism, the less are its fermentations reduced by oxygen. It appears that fermentation is markedly reduced by oxygen only if the organism can utilize substantial amounts of energy derived from oxidative metabolism. This ability seems to be lacking in worms which possess a predominantly anaerobic type of metabolism. In these organisms respiration and fermentation are independent of each other. This would explain why their rates of fermentation are not, or are only slightly, decreased regardless of whether or not the parasites respire, and why inhibition of the oxygen uptake of *Schistosoma mansoni* does not result in a compensatory increase of glycolysis. Therefore, parasitic worms could be classified into two groups: 1) Those organisms in which respiratory metabolism has little or no effect on their rate of fermentation. This group does not appear to require or to utilize energy liberated by oxidative reaction. 2) Those helminths which utilize oxidative energy. In these organisms inhibition of respiration results in an increase in fermentation and eventually in their death. Investigations of a larger number of parasitic helminths should determine the validity of such an hypothesis and classification.

Anaerobiosis of many parasitic helminths and of other invertebrates is followed by a temporary rise in the oxygen uptake after aerobic conditions are re-established (18, 181, 86, 70-72, 47, 48, 114, 203, 210, 211). This repayment of the oxygen debt incurred during anaerobiosis has been considered as a general phenomenon among invertebrates. However, the respiration rates of *Litomosoides carinii* and of *Schistosoma mansoni* are the same before and immediately following anaerobiosis (26, 27). It has been assumed that during anaerobiosis products of fermentation accumulate and that these are oxidized as soon as aerobic conditions are re-established (210). Such an interpretation would explain the absence of a post-anaerobic increase of the oxygen uptake of the filariae and of the schistosomes; the major product of fermentation of both organisms is lactic acid and addition of this substance to the medium produces no increase in their oxygen uptake. It has not been established which products of fermentation are oxidized by organisms in which a post-anaerobic increase of respiration occurs.

Little is known about the intermediary carbohydrate metabolism of parasitic helminths. Evidence has been obtained that carbohydrate metabolism of *Litomosoides carinii* is associated with phosphorylations. When this organism is incubated aerobically in a medium containing radioactive phosphorus as inorganic phosphate, incorporation of  $P^{32}$  in the organic acid soluble phosphate fraction is at least 3.5 times higher in the presence of glucose than in its absence (29). If aqueous extracts of *Ascaris lumbricoides* are incubated in the presence of fluoride esterification of inorganic phosphate can be demonstrated (151). Extracts of male *Schistosoma mansoni* exhibit high zymohexase and glyceraldehyde phosphate dehydrogenase activity (27). These observations indicate that lactic acid formation in these three parasites may proceed according to the Embden-Meyerhof scheme. Further work is necessary to establish

this with certainty. It also remains to be determined whether the various types of phosphatases, which have recently been found in a number of parasitic helminths (30, 135-137, 149), play a significant role in the carbohydrate metabolism of these organisms. In several respects the metabolism of pyruvic acid by *Lilomosoides carlinii* is similar to that of certain mammalian tissues and bacteria. Anaerobically the filariae convert two moles of pyruvate to one mole of lactate, one mole of acetate and one mole of CO<sub>2</sub> (26). This anaerobic dismutation of pyruvate also occurs in brain (225) and in a number of bacteria (107, 108). Aerobically, the filariae metabolize pyruvate to acetate, lactate, acetylmethylcarbinol (16), and to other as yet unidentified product or products. Suggestive evidence has been obtained indicating that oxidation of carbohydrate by small intestinal nematodes may involve the tri-carboxylic acid cycle (152). In the latter organisms, in contrast to the filariae (26), oxidation of acetate occurs (152).

More than 45 years ago, Weinland (228) demonstrated the formation of valeric acid from glycogen by extracts from *Ascaris lumbricoides*. In addition, several statements in the literature refer to the 'glycolytic' activity of cell-free material originating from parasitic helminths (62, 116), indicating the presence of enzymes which catalyze the removal of glucose. While the remarkable advances in the field of intermediary carbohydrate metabolism of vertebrates, yeast and bacteria were initiated by the use of cell-free extracts, it is astonishing to note that similar methods have not been applied to parasitic helminths for the investigation of the intermediary reactions and the enzyme systems concerned with the conversion of carbohydrate to volatile and higher fatty acids.

#### LIPID METABOLISM

Experimental data obtained by chemical determination of lipids indicate that higher fatty acids are not metabolized to any significant degree by the large parasitic helminths inhabiting the intestinal tract and the bile ducts. Weinland (227) found that no decrease in the lipid content of *Ascaris* occurs when this organism is kept in a non-nutrient salt solution for several days. These observations were confirmed by Schulte (161) and by Von Brand (195). Subsequently, the latter author (201) demonstrated that starvation of *Ascaris* for five days under aerobic conditions results in a small loss of fat. However, at the end of the experimental period, the sum of the fat excreted with the eggs and of the fat of the *Ascaris* body was identical with the initial fat content of *Ascaris* (201). Similar experiments with *Fasciola hepatica* (192, 233) and with *Moniezia expansa* (194) have revealed that the sum of the lipid excreted and present in the worms after starvation was higher than the initial lipid content of the organisms. This increase can be explained by the fact that higher fatty acids are products of the carbohydrate metabolism of these two parasites (see preceding section). However, the fat content of the bodies of the helminths remained constant throughout the experimental period (233, 194). Mueller (127) has reported a decrease in the fat content of *Ascaris* tissue kept in the perienteric fluid of this organism for eight days. On the basis of this frequently quoted observation he concluded that fat oxidation occurs in *Ascaris*. In these tissue cultures, fat was not determined chemically, but only by histological staining techniques. Results obtained in this

manner are difficult to evaluate on a quantitative basis. Furthermore, even if the reduction in stainable fat reflected a true decrease in the lipid content of *Ascaris* tissue, this would not prove that oxidation of fat had occurred. It is quite possible that this material merely passed into the surrounding fluid.

Several parasitic helminths possess esterase activity since fatty acid esters of glycerol and acetylcholine are hydrolyzed by extracts from these organisms. Lipase and choline esterase activities of *Fasciola hepatica*, of *Taenia taeniformis* and of *Taenia pisiformis* were found to be much lower than those of free living platyhelminths (135). Lipase activity has been detected also in *Strongylus edentatus* (146), *Ascaris* (146) and in two acanthocephala (30); cholinesterase activity in *Parascaris equorum* (8), in *Taenia crassicolis* (7) and in *Dypylidium caninum* (7). The substrate specificity and the physiological significance of these esterases have not been determined.

Several lipids with unusual properties occur in parasitic helminths. Flury (62) found that the major portion of the unsaponifiable fraction of *Ascaris* consists of a wax-like material which had the characteristics of an alcohol of high boiling point and which he called 'ascaryl alcohol.' This was studied in greater detail by Schulz and Becker (162) who isolated it both from *Ascaris lumbricoides* and from *Parascaris equorum*. On the basis of the elementary analysis of the pure compound (melting point 84° C.), these authors ascribe to it the formula  $C_{22}H_{44}O_4$ . Only two of the oxygen atoms could be accounted for by hydroxyl groups. The other two participate in ether linkages. The exact structure of this interesting compound has not been elucidated. It is present in the eggs of *Parascaris equorum* (54), but it also has been isolated in large quantities from male *Ascaris lumbricoides* (209).

Lesuk and Anderson (120) isolated from *Cysticercus fasciolaris* a cerebroside which contains as its nitrogenous base dihydrosphingosin, but no sphingosin, the usual constituent of cerebrosides. The same authors found that fatty acids of lecithin isolated from this organism were completely saturated and consisted mainly of palmitic acid. Data on the phospholipid content of *Ascaris lumbricoides* have been obtained recently by Rogers (151). According to Smorodinzev and Bebechine (169) the lipids of *Taeniarrhynchus saginatus* contain an extremely large amount of unsaturated fatty acids. Since the segments of this cestode partially disintegrate in the intestinal tract and since unsaturated fatty acids produce hemolysis, these authors suggest that absorption of these acids might cause the anemia observed in infestations with cestodes. This hypothesis appears to be supported by the experiments of Wardle and Green (223) who produced anemia by feeding the fatty acids isolated from two tapeworms, *Moniezia expansa* and *Diphylllobothrium latum*.

#### PROTEIN METABOLISM

Only a few investigations on the protein metabolism of parasitic helminths have been carried out. Weinland (232) found that the daily excretion of nitrogen by *Ascaris lumbricoides* amounts to 15 to 20 mg/100 gm. wet weight. This was confirmed by Von Brand (195). According to this author, nitrogen excretion was the same under aerobic and anaerobic conditions and approximately 30 per cent of the excreted nitrogen originated from eggs and egg shells. Weinland (232) showed that one-third of the excreted nitrogen consists of ammonia. The total nitrogen excreted by *Fasciola hepatica* (233) and by *Moniezia expansa* (194) also has been determined.

Urea does not appear to be a product of the protein metabolism of *Ascaris* or of other parasitic helminths. Chitwood (36) has found that 24 hours after *Ascaris* has been removed from its host, the excretory fluid of the parasite contained no urea. Ammonia, but neither urea nor uric acid, is present in *Fasciola hepatica*, *Moniezia benedini* and *Taenia pisiformis* (188). No urease, allantoinase or allantoinase activity could be detected in *Fasciola hepatica* (188, 61). Van Grembergen and Pennoit-de-Cooman (188) also have reported that extracts of *Moniezia benedini* and of *Taenia pisiformis* have no urease activity. On the other hand, these cestodes, as well as *Fasciola hepatica*, have a high arginase activity (188). According to Schopfer (160) uric acid is formed by *Cysticercus tenuicollis* because the liquor of the cyst contains 6.5 times more uric acid than the extracellular fluid of the host.

Extracts possessing proteolytic activity have been prepared from a great number of parasitic helminths (105, 179, 1, 62, 63, 116, 165, 154, 36, 191, 147, 135). Optimal proteolytic activities of such crude extracts were observed between pH 2.2 and 8.0 and varied with the organism. In some of them several pH optima were noted indicating the presence of more than one proteolytic enzyme (135). Since none of these enzymes has been purified to any significant degree no definite comparison with the properties of proteolytic enzymes from other animals is possible. Rogers (147) has demonstrated that the proteolytic activity of extracts from *Strongylus edentatus* is 5 to 8 times higher than that of extracts of *Ascaris*. The former nematode ingests blood and tissue while the latter receives its food supply from the intestinal contents in which proteins are already partially digested by the host.

Histochemical evidence has been reported indicating that ribonucleo-proteins are synthesized during fertilization of eggs of *Parascaris equorum* (133).

A characteristic property of cestodes is their low protein content (166 to 168). Kent and Macheboeuf (101 to 104) have observed interaction of protein fractions from the cestode *Moniezia expansa* with glycogen and with an alcohol-soluble material which gives a positive Pettenkoffer test. This color reaction is obtained with bile acids, a great number of other steroids (215, 156), other aromatic compounds (60) and with some aliphatic alcohols and acids (60). The nature of the interaction of proteins of *Moniezia* with glycogen and with other substances remains open to further investigations.

The external cortical layer of the cuticle of *Ascaris* has been found to consist of keratin (35). This protein was detected also in the hooks of *Cysticercus fasciolaris* (43). Chitin has been found to occur in the egg membranes of *Parascaris equorum* (53, 238), of *Ascaris lumbricoides* (37, 158), of pinworms (97), and of *Macracanthorhynchus hirudinaceus* (200).

#### RESPIRATORY ENZYMES

A number of investigations on the enzymatic mechanisms catalyzing the transfer of hydrogen in parasitic helminths have been carried out. Gourevitch (73) observed that *Fasciola hepatica* and *Parascaris equorum* the metabolism of which is predominantly anaerobic contain considerably less flavin than insects and the tissues of vertebrates. Since in bacteria the flavin concentration is much higher in anaerobic than in aerobic organisms (221) the author concluded that this rule does not apply to metazoa.

Circumstantial evidence is available that flavine enzymes are involved in the hydrogen transfer mechanisms in parasitic helminths (34, 3). Pennoit-de-Cooman and Van Grembergen (135) have demonstrated the enzymatic reduction of methylene blue by extracts of *Fasciola hepatica*, *Taenia pisiformis* and of *Moniezia benedini*. Reduction of methylene blue was greatly accelerated by the addition of succinate (135). Similar findings were made with *Ascaris lumbricoides* by Laser (114). Since flavin enzymes react with methylene blue it is possible that these enzymes catalyze the reduction of methylene blue observed in these experiments. They also indicate that the parasites studied possess high succinic dehydrogenase activity.

Laser has shown that, in the presence of atmospheric oxygen, enzymatic dehydrogenations in *Ascaris lumbricoides* result in the formation of hydrogen peroxide (114). This substance inhibited the respiration of the organism. Since the worms contain little catalase (123, 114), hydrogen peroxide accumulates when the nematodes are incubated in air. Removal of hydrogen peroxide by the addition of catalase resulted in an increase in the oxygen uptake of this organism (114). Extremely low catalase activity was found also in *Moniezia benedini*, *Taenia pisiformis* and *Cysticercus pisiformis* (135).

Observations of Harnisch (87) indicate that the body fluid of *Ascaris lumbricoides* and of several cestodes (*Moniezia expansa*, *Abothrium rugosum* and *Abothrium crasum*) contain one or several factors essential for optimal respiration of these organisms. According to this author the low oxygen uptake of washed tissue mince is greatly increased by the addition of the body fluid of the parasites while the body fluid alone has no respiratory activity. It would be of interest to determine whether the body fluid supplies substrates or a coenzyme activating respiratory mechanisms of these helminths.

Keilin (98) detected in *Ascaris lumbricoides* the spectral absorption bands of cytochrome C, A and B. Similarly, the approximate absorption bands of cytochrome C were present in *Diphyllobothrium latum* (67), *Triaenophorus lucii* (67), *Allassostoma magnum* (236), and *Camallanus trispinosus* (236), and those of cytochrome B and C were found in *Moniezia benedini* (187) and in *Fasciola hepatica* (189). Treatment of a tissue mince of *Moniezia benedini* with hydrosulfite, hydrogen peroxide or pyridine had the same effects on the spectral characteristics of this material as on those of cytochrome C and B (187). Similarities of the absorption spectra of the cytochromes with those found in the parasites do not necessarily prove that the latter contain the cytochrome system. The observed optical properties of the worms may merely indicate that these organisms contain some hematin-like pigments. Whether these pigments have the same enzymatic properties as the cytochromes has not been determined. As pointed out by Keilin and Hartree (100) the most fundamental property of cytochrome C is its activity as a biological oxidation catalyst. Since homogenates of filariae had no cytochrome C activity (27) when tested with cytochrome oxidase and hydroquinone (76), convincing evidence for the presence of cytochrome C in parasitic helminths is lacking.

Nor has it been demonstrated that a major portion of the hydrogen transport of any parasitic helminth is mediated by cytochrome oxidase. In fact, several observations indicate that this enzyme might not play a significant role in a number of these organisms.

The effect of inhibitors of cytochrome oxidase (99) on the respiration of parasitic worms has been studied. Respiration of the larvae of *Trichinella* is strongly inhibited by low concentrations of cyanide (171). High concentrations ( $2.5 \times 10^{-3}$  to  $1 \times 10^{-2}M$ ) of cyanide and azide inhibit the respiration of *Eustrongylides* larvae to an extent of 72 and 85 per cent, respectively, but with lower concentrations this effect is much less pronounced (207). A reduction of the oxygen uptake of *Diphyllbothrium latum* to 15 per cent of its control value was observed only with very high cyanide concentrations ( $5 \times 10^{-3}$  to  $1 \times 10^{-2}M$ ) (67, 68). Incomplete respiratory inhibition by high concentrations of cyanide was also observed in *Taenia taeniformis* (237), in *Moniezia benedini* (187) and in the following intestinal nematodes: *Ascaridia galli*, *Nematodirus Spp.* and *Nippostrongylus muris* (150). Respiration of *Neoplactana glaseri* (150), of *Lilomosoides carinii* (26) and of *Schistosoma mansoni* (27) was inhibited completely by considerably lower cyanide concentrations. In concentrations up to  $1 \times 10^{-2}M$  cyanide did not inhibit the oxygen uptake of *Ascaris lumbricoides* (114, 190). However, this apparent absence of inhibition might be due to the removal from the reaction mixture of oxaloacetic acid by cyanide through cyano-hydrine formation since oxaloacetic acid inhibits the respiration of *Ascaris* (114). Reduction of the oxygen uptake of parasitic helminths by cyanide may merely indicate the presence of a heavy metal containing respiratory enzyme, but not necessarily that of cytochrome oxidase. This is illustrated by the following example: *Lilomosoides carinii*, the respiration of which is more sensitive to cyanide than that of most other parasitic helminths, possesses no cytochrome oxidase activity since homogenates of this organism do not oxidize reduced cytochrome C (27), the specific substrate of cytochrome oxidase. While this enzyme is inactivated by carbon monoxide in the absence of light (220) respiration of *Moniezia benedini* is inhibited to a slight degree (187) and that of *Diphyllbothrium latum* is not affected at all under these conditions (67, 68). The fact that paraphenylenediamine increases the respiration of larval *Eustrongylides* (207) and of *Moniezia benedini* (187) does not prove the presence of the cytochrome-cytochrome oxidase system in these organisms, because, conceivably, this labile substance could also be oxidized by other enzymes. Since the respiration of *Ascaris* results in the formation of hydrogen peroxide (114), it does not appear likely that cytochrome oxidase is involved in the hydrogen transfer mechanisms of this parasite because reduced cytochrome oxidase reacts with atmospheric oxygen to form water and not hydrogen peroxide.

#### RESISTANCE TO DIGESTION

Parasitic helminths are not digested in the intestine despite an abundance of proteolytic enzymes in this habitat. Neither are living intestinal nematodes or cestodes digested by trypsin (32). However, rapid digestion occurs if these organisms have been killed (32). The mechanism by which intestinal helminths are protected from digestion may be similar to that which prevents the destruction of many other living cells (e.g., those of the gastro-intestinal mucosa) by proteolytic enzymes. Fermi (56) and Northrop (128) demonstrated that when living earthworms, arthropods, fish, or protozoa were placed in solutions of trypsin or pepsin, no digestion and no decrease in the enzyme content of the solution occurred. However, when killed by heat, the same organisms removed a considerable amount of these proteolytic en-

zymes and were rapidly digested. Northrop (128) concluded, therefore, that the membranes of living cells are not permeable to pepsin and trypsin.

In round worms this general impermeability of living cells to digestive enzymes is enhanced by the external cuticle which is resistant to digestion. This cuticle is particularly well developed in parasitic nematodes. As demonstrated by the observation of Filipjev (58) intra-vitam stains, e.g., methylene blue, penetrate the cuticles of free-living worms, but not those of *Ascaris*, the external cuticular layer of which consists of a keratin (62) that is not digested by trypsin or pepsin (35). The protective role of the cuticle against digestive enzymes was demonstrated in 1878 by Fredericq (66), who placed intact *Ascaris marginalis* in pancreatic juice. No digestion occurred in this medium. However, when the parasites were cut in several fragments all tissues with the exception of the cuticle were digested rapidly. De Wale (49) made similar observations with living *Taenia saginata* which was digested by pepsin or trypsin only if a slight incision was made in the proglottis or the scolex. It is evident, therefore, that the cuticle of parasitic helminths is impermeable to digestive enzymes.

Another factor which may play an important role in the protection of parasitic helminths against digestion is the presence of trypsin and pepsin inhibitors in these organisms. This was discovered in 1903 by Weinland (230), who found inhibitors of the proteolytic activities of trypsin and of pepsin in extracts of *Ascaris* and of *Taenia*. The observations were confirmed by Fetterolf (57) and Tallquist (179) for tapeworms and by Hamill (78) for *Parascaris equorum*. The latter author showed also that the trypsin inhibitor of *P. equorum* is dialyzable, heat-stable in acid and neutral solutions, heat-labile in alkali and that it is precipitated only by high concentrations of alcohol. He concluded that the inhibitor is not a protein, but that its properties are similar to those of a polypeptide. Similar observations were made by Harned and Nash (79). Stewart (175) found that nematodes living in the gastrointestinal tract of sheep (46) contain a pepsin inhibitor. He ascribed the decreased digestion of proteins by lambs heavily infested with these parasites to the action of this inhibitor (174). According to Sang (154) the trypsin inhibitor also has the properties of a proteolytic enzyme. Since no extensive fractionation and purification of the inhibitor was attempted by this author, his findings may signify merely that the inhibitor had not been separated from proteolytic enzymes. The same difficulty was encountered by Von Bonsdorff (191), who failed to detect any inhibition of trypsin or of pepsin by extracts of *Diphyllolobothrium latum* and of *Taenia saginata*. These extracts had marked proteolytic activity and, therefore, may have masked the presence of inhibitors when added to trypsin or pepsin. Furthermore, Von Bonsdorff (191) makes no statement as to how his extracts were prepared. As already noted by Weinland (230) and confirmed by Mendel and Blood (126), the trypsin and pepsin inhibitors of parasitic helminths are quite insoluble and can be extracted only by very thorough grinding. The purification and isolation of a trypsin inhibiting polypeptide from *Ascaris* by Collier (38) has supplied definite evidence for the existence of such a compound. It is similar in almost every respect to the trypsin inhibitors crystallized from beef pancreas by Kunitz and Northrop (112) and isolated from blood serum (55) by Schmitz (159). However, in contrast to the latter two substances, inhibition of trypsin activity by the polypeptide from *Ascaris* was not

reversed at a low pH (38). In view of these similarities, it would be of interest to determine whether the trypsin inhibitor of *Ascaris* is supplied by the host or whether it is synthesized by the parasite itself. Since the pepsin inhibitor present in parasitic helminths has not yet been purified, no comparison of its properties with those of the pepsin inhibitor crystallized by Herriot (129) from pancreas is possible.

In contrast to their marked anti-tryptic and anti-peptic activity extracts from parasitic helminths have no inhibitory action on papain (126). Living *Ascaris* are digested by this (15) and other proteolytic enzymes of vegetable origin such as bromelain (14), present in pineapple juice, and by ficin (217, 145, 216, 6) isolated from the sap of *Ficus laurifolia*.

It can be concluded, therefore, that the resistance of intestinal helminths to digestion may be produced by at least three factors: 1) the impermeability of living cells to digestive enzymes; 2) the impermeability of the external cuticle; and 3) the action of trypsin and pepsin inhibitors of the parasites. At present, the relative significance of each of these factors for the protection of the organisms against digestion cannot be ascertained.

#### EFFECT OF ANTHELMINTIC AGENTS ON THE METABOLISM OF PARASITIC WORMS

Routine testing programs, in which large numbers of unrelated compounds have been assayed for anthelmintic activity, have constituted the principal approach to the experimental chemotherapy of helminthic infestations up to the present time. In the course of such empirical studies, little knowledge has been gained concerning the mechanism of action of existing anthelmintic agents and the basic physiology of the parasites involved, despite the great potential value that such information would have in suggesting new and more logical approaches to this problem. Attention has been called to this situation in a recent review by Wright (240). The fundamental question as to whether any anthelmintic drug in use at the present time exerts its effect by inhibiting essential metabolic processes of the parasites against which it is effective has not been investigated to any significant extent.

It has been shown recently, however, that two groups of compounds, possessing anthelmintic activity in infested animals, produce their effects by inhibiting certain metabolic activities of the parasites. Recent experimental evidence indicates that the marked chemotherapeutic effectiveness of the cyanines against the filariasis of the cotton rat is based on their inhibitory effect on the oxidative metabolism of *L. carinii* (26, 234, 138). The administration of cyanine dyes has little or no effect on the survival *in vivo* of *S. mansoni* (24, 25, 141) and of certain larger helminths inhabiting the intestinal and biliary tracts (77, 141). These refractory organisms exhibit little or no dependence on respiration (see above). The cyanines, however, do possess activity against *Ancylostoma caninum* (77), which is believed to have an essential requirement for oxidative metabolism (198, 199). Thus, inhibition of respiratory processes appears to be the basis for the chemotherapeutic action of the cyanines against *L. carinii* and *A. caninum*; at the same time, it probably explains the lack of a chemotherapeutic effect against worms whose metabolism is predominantly anaerobic.

By way of contrast, the rate of glycolysis of *S. mansoni* is inhibited by a number of naphthoquinones which, however, have little effect on the respiration of the trema-



todes (23-25). These compounds have slight but definite chemotherapeutic activity against *S. mansoni* in mice, because they potentiate the effect of very small and otherwise ineffective doses of 'Fuadin' (23, 24). The rate of glycolysis of schistosomes removed from mice treated with this combination is markedly reduced (23). These observations indicate that the chemotherapeutic activity of such naphthoquinones is based on their ability to decrease the rate of glycolysis of *Schistosoma mansoni*. The fact that this effect is markedly decreased by serum albumin explains (23, 25), at least in part, the low chemotherapeutic activity *in vivo*. 'Fuadin', a much more effective chemotherapeutic agent in this condition when large doses are employed, inhibits glycolysis of schistosomes to a lesser degree than oxygen uptake (24, 25). Thus, this compound is quite effective in reducing *aerobic* metabolism, which at best plays a minor role in the survival of the worms; inhibition of essential glycolytic processes, on the other hand, occurs only with concentrations of the drug approaching those capable of producing injury to the host. These findings parallel the well-known and regrettable fact that clinically effective doses of 'Fuadin' frequently produce toxic reactions in the human host. The reversal of the anti-metabolic effects of 'Fuadin' against *S. mansoni* by BAL (British Anti-Lewisite) suggests that the inhibitory effects of the antimonial on glycolysis are due to inactivation of sulfhydryl enzymes (27a). The enhancement of the anti-glycolytic effect of p-chloromercuric benzoate by BAL *in vitro* (27a) is an interesting paradox. The possibility that a combination of these two agents might exert a chemotherapeutic effect against *S. mansoni in vivo* is an intriguing one, especially because BAL reduces the toxicity of mercury for the mammalian host.

As demonstrated recently by Baldwin (10, 12), low concentrations of santonin, an effective agent against *Ascaris lumbricoides in vivo*, have a selective toxic action on the ganglion located in the 'nerve ring' of this worm, and hexylresorcinol, an equally effective but less toxic ascaricide, has a direct paralyzing effect on its muscle. Trim (185) demonstrated that hexylresorcinol readily penetrates the cuticle of the ascarids. Subsequently, several factors affecting the rate of diffusion of hexylresorcinol and of other phenols into nematodes have been studied (186, 148, 4). Since the action of the anthelmintics is, in some cases at least, a specific one, involving specific parts or organs of the worms, the question as to the mechanism by which these localized effects are produced presents itself.

The evidence discussed in this review indicates that the biochemical characteristics of parasitic worms vary greatly from one species to another. In view of these differences, which are much more pronounced than those existing among vertebrates, it is not surprising that many drugs which are highly effective against one particular type of parasite, are completely inactive against others. Even among parasitic worms possessing many morphological similarities, profound metabolic differences may prevail. This is well illustrated by the marked chemotherapeutic activity of cyanine dyes against the filarial worm, *Litomosoides carinii*, on the one hand (234, 26, 138), and their complete lack of activity against two other adult filarial species, *Dirofilaria immitis* (141) and *Wuchereria bancrofti* (155a). It was not possible to study the latter parasite, which resides in the lymphatic system of its human host, *in vitro*; hence the possibility exists that the cyanine dyes were ineffective because of their failure to enter the lymph following their intravenous injection into man.

In dogs, however, comparable doses of cyanine dyes administered by the same route bestowed on the lymph the ability to inhibit the respiration of *L. carinii* *in vitro* (140). *Dirofilaria immitis*, of course, was subjected to very high concentrations of cyanine dyes *in vivo*, since the drug was injected into the vascular system, the actual site of occurrence of the infestation, yet the parasite survived. The metabolic differences between *L. carinii* and *W. bancrofti* are reflected also in the fact that 1-diethylcarbamy1-4-methylpiperazine ('Hetrazan') possesses marked chemotherapeutic activity against the latter parasite *in vivo* (155), while its effect on the former was of a very low order, both *in vivo* and *in vitro* (91). In the case of yet another tissue nematode, *Dracunculus insignis*, low concentrations of the cyanines produce a considerable reduction of the oxygen uptake, but no compensatory increase in the rate of glycolysis (28). This suggests that *Dracunculus insignis* also is much less dependent on oxidative metabolism than is *Lilomosoides carinii*.

Such profound biochemical variations may be of considerable importance with regard to the development of new anthelmintic drugs. They may afford opportunities to inhibit essential metabolic reactions of the parasite, which may not occur in the host, or which, if present, may not be essential to its functional integrity. Furthermore, since morphological and taxonomic similarities of parasitic worms do not necessarily indicate biochemical similarities of parasitic worms, the metabolic characteristics of the particular organism against which chemotherapy is directed must be investigated rather than that of related species of greater availability. Once the essential metabolic reactions of a parasite are known, attempts to block such processes may be initiated.

Up to the present time only catabolic reactions of parasitic helminths have been studied. As pointed out by Hotchkiss (95), the anabolic metabolism of pathogenic organisms may prove to be even more vulnerable to inhibition. Since such reactions proceed at relatively slow rates, the organisms must be cultured aseptically outside the host for prolonged periods of time. In the past 15 years considerable progress has been made in establishing such conditions with parasitic worms, although this has not been possible as yet with media of known chemical composition.<sup>3</sup> Once this latter goal has been achieved, a study of the exact nutritional requirements of parasitic helminths will be feasible. Furthermore, under such conditions, experiments can be designed, in which attempts are made to block the utilization of essential metabolites by competitive and non-competitive inhibitors. It is quite possible that the essential requirements of a given parasite for several factors may differ qualitatively and quantitatively from those of the host. Thus, under proper conditions of dosage, prevention or reduction of the utilization of such factors might injure the parasite, but not its host.

The concept of inhibiting the essential metabolic reactions of parasitic helminths, theoretical at the present time, will increase in feasibility as additional information is acquired in regard to the specific chemical reactions involved. The number of points of attack likewise will increase on this basis. It is to be expected, however, that inhibitors effective *in vitro* frequently may not be active *in vivo*, due to inacti-

<sup>3</sup>Since this problem is outside the scope of this review, the reader is referred to reviews on this subject by Hoeppli *et al.* (94), by Hobson (93), and by Smyth (170), and to recent work of Stephenson (172).

vation, unfavorable distribution, metabolic alteration, slow absorption, or rapid excretion by the host. In some instances, the host's ability to exert such influences may be decreased by chemical modification of the metabolic inhibitor in a way which does not alter the intrinsic activity of the compound *in vitro*, as well as its toxicity for the host. These latter factors represent large problems in themselves and practical results may not occur in the immediate future. Nevertheless, it is believed that advances in the field of comparative biochemistry of parasitic worms can form the basis for a more rational approach to the chemotherapy of helminthiasis, an approach which should supplement, and eventually may even replace the presently prevailing empirical methods.

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# DETERMINATION OF AMINO ACIDS BY MICROBIOLOGICAL ASSAY<sup>1</sup>

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IT WAS REPORTED in September 1943, by Kuiken *et al.* (79) of the Texas Agricultural Experiment Station that nine amino acids (cystine, glutamic acid, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, and valine) were essential for the growth of *Lactobacillus arabinosus* 17-5 and that three amino acids (isoleucine, leucine and valine) could be determined rapidly and accurately by microbiological techniques similar to those utilized for vitamin assays. In December of that year these workers (80) described their microbiological procedure and reported the results of assays of gelatin and four food products for these three amino acids. The basal medium employed was that developed by Snell and Wright (162) in 1941 for the determination of nicotinic acid modified to contain *p*-aminobenzoic acid, a tomato juice eluate, and 17 amino acids in place of acid-hydrolyzed casein. During the intervening years microbiological methods have been elaborated in the laboratories of Lyman, Stokes, Snell, Elvehjem and other investigators for the determination of nearly all of the naturally occurring amino acids. Many of the amino acids have been determined in purified proteins, foods, blood, urine, sweat, spinal fluid, hormones, enzymes, viruses, bacteria and bacterial toxins, fungi, plant products and other types of biological materials. It is gratifying, therefore, that, through microbiology, significant advances have been made in our knowledge of amino acids and proteins in biology and medicine.

Achievements of such magnitude and apparent value are not uncommon in the annals of biological science, and, as in comparable cases, there is a background of notable accomplishments by many earlier workers without which present successes would have been impossible. That the assay potentialities of micro-organisms, virtually unknown a decade ago either for vitamins or amino acids, have been revealed in this relatively short time is of course only the culmination of countless experiments performed since microbes were first observed by von Leeuwenhoek (83) in 1685. We may be reminded, however, that modern investigations were not initiated until about 1860 by Pasteur who discovered that bacteria are causal agents of fermentation, putrefaction and disease.

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<sup>1</sup> Snell (146, 153, 155) has published excellent review articles on microbiological methods for the estimation of amino acids. Doctors M. N. Camien and L. B. Rockland have rendered valuable assistance in the preparation of this paper, which was presented essentially in the present form on August 9, 1948 at the Gordon Research Conference, New London, New Hampshire. Dr. Clayton D. Sander, Eastern States Farmers' Exchange, Buffalo, N. Y., rendered valuable assistance in checking the data and calculations. The author is indebted to the Nutrition Foundation for financial aid in support of his microbiological research work and for payment of extra publication costs. Paper 50. For Paper 49, see Murphy and Dunn, in press.



During several decades Pasteur, Liebig and other outstanding investigators of the time concerned themselves with the metabolism of yeasts, particularly fermentation, in attempting to find a chemical basis for the physiological activity of the cell. Because of the theoretical and practical importance of yeasts and fermentative processes, such studies have been carried on increasingly since Pasteur's day by many workers mentioned in reviews of these topics by Harden (52) and other authors (1, 58, 84, 106, 108). Following the isolation of *Bacillus anthracis* about 1850, the bacterial forms responsible for scarlet fever, meningitis, botulinism, tetanus, tuberculosis and other diseases were identified prior to 1900. Nencki (107) and other investigators, referred to in reviews by Leach (82) and other authors (18, 140, 175), have determined the chemical composition of microbial cells in the search for clues to the causes of diseases and the pattern of evolutionary processes. Extensive morphological and physiological studies have been made, also, leading to the classification

TABLE 1. COMPOSITION OF BASAL MEDIA EMPLOYED BY PASTEUR AND OTHER EARLY WORKERS

CONSTITUENT	BASAL MEDIUM FOR YEAST		BASAL MEDIUM FOR <i>B. typhosus</i> AND OTHER ORGANISMS
	Pasteur, 1860	Wildiers, 1901	Uchinsky, 1893
Sucrose.....	X	X	
Ammonium tartrate or lactate.....	X		X
Yeast ash.....	X		
Glycerol.....			X
NaCl or KCl.....		X	X
CaCl <sub>2</sub> or CaCO <sub>3</sub> .....		X	X
MgSO <sub>4</sub> .....		X	X
K <sub>2</sub> HPO <sub>4</sub> or Na <sub>2</sub> HPO <sub>4</sub> .....		X	X
Sodium aspartate.....			X
NH <sub>4</sub> Cl.....		X	

of bacterial types as given in the Bergey's *Manual of Determinative Bacteriology* (6) and other treatises.

The elaboration of synthetic media on which bacteria will grow has been a problem of major concern even to the earliest workers. The conclusion of Pasteur (116) in 1860 that yeasts could be cultured on an aqueous solution containing only chemically-defined substances was unacceptable to Liebig and, apparently, was disproved by Wildiers (178) in 1901. The latter worker found that yeast would not grow unless an unknown factor, which he named 'bios'<sup>2</sup> in addition to sucrose and the salts shown in table 1 was present in the culture medium. On the other hand, Uchinsky (172) had shown earlier (1893) that typhoid, cholera, diphtheria and certain other organisms could be cultivated on a synthetic medium of the composition shown in table 1. Of interest in this connection is the recent report of Wooley and Sebrell (184) of a microbiological procedure for the determination of tryptophan with a particular strain of typhoid bacillus grown on a synthetic basal medium containing

\* The 'bios' problem has been reviewed by Tanner (171).

besides 15 amino acids only glucose, sodium chloride, magnesium sulfate and potassium dihydrogen phosphate.

During the past half century numerous workers interested primarily in the metabolism of microorganisms have grown bacteria on synthetic media varying little in composition from the mixtures employed by Uschinsky. Types of organisms which have been cultured include *Escherichia coli* (36, 44, 141), *Corynebacterium diphtheriae* (171), *Bacillus lactis aerogenes* (56), *Streptococcus faecalis* (145), *Bacillus pyocyaneus* (78), *Mycobacterium tuberculosis* (10, 22, 78, 86, 88, 135, 136, 143, 169), *Azotobacter chroococcum* and other strains (12, 13, 48, 75) of *Azotobacter*, and various related bacterial forms. Since it has been observed that certain bacteria grow poorly, if at all, on media of composition as simple as that of the original or modified Uschinsky formula, improvement in the nutritional quality of culture media has been an objective of numerous workers since the time of Pasteur. Natural materials or extracts of naturally-occurring products found to be effective supplements of synthetic media include yeast, blood, peptones, beef muscle, liver, fish, urine, manure, and grass. Owing to the complexity of such mixtures slow progress was made until recently in determining the identity of any components which might be essential for the growth of bacteria.

The bacteria of most importance to the present discussion are the *Lactobacillus* genus of the Lactobacillae and the *Streptococcus* and *Leuconostoc* genera of the Streptococcae. These non-motile, non-spore forming, Gram-positive cocci and rods are essentially anaerobic organisms which produce lactic acid, and in some cases other organic acids, from carbohydrates. These bacteria are of practical importance because of their prominent role in the manufacture of sauerkraut, the production of silage, the aging of cheese, the brining of olives and pickles, the spoilage of beer, the decay of the teeth and other processes. The names of some common types of lactic acid bacteria are given in table 2.

It was first observed in 1857 by Pasteur (115) that lactic acid is produced by bacteria which ferment sugar and the first true lactic acid bacterium, *Streptococcus lactis*, was isolated in 1873 by Lister (85) who employed a basal medium essentially the same as that (table 1) described by Pasteur (116) in 1860. Most early workers, including Kayser (64) in 1894, grew bacteria on media containing peptonized milk, peptones, gelatin, yeast or other protein supplement and the standard types of media shown in table 3 were suggested by Henneberg (55) in 1903.

The most fundamental studies of the lactic acid bacteria made by early workers were those of Orla-Jensen who initiated (110) his investigations on the nutrition, morphology and physiological characteristics of micro-organisms in Copenhagen about 1898. Orla-Jensen's classical treatise (111) entitled, "The Lactic Acid Bacteria," was published in 1919 and his book on dairy bacteriology (112) in 1931. Of particular interest in relation to the topic under discussion are his studies (113) in 1936 on growth factors in milk and peptones and on the vitamin and nitrogen requirements of lactic acid bacteria. It was established that unknown factors are required for the growth of *bulgaricus*, *helveticus*, *casei*, *lactis* and other *Lactobacilli* and it was concluded that riboflavin and probably pantothenic acid are essential-growth substances for these organisms. Advantage of these discoveries was soon taken by other work-

ers in elaborating 'synthetic' diets for lactic acid bacteria containing these two vitamins. This milestone in nutritional research could not have been reached easily at an earlier time even though the vitamin concept had been accepted as valid since the experiments of Hopkins (59) in 1912. Although the presence of water-soluble substances in milk which promote the growth of young rats was noted by Osborne

TABLE 2. HISTORY OF DISCOVERY OF COMMON LACTIC ACID BACTERIA<sup>1</sup>

ORGANISM	DISCOVERER	DATE
<i>Streptococcus lactis</i> .....	Lister	1873
<i>Leuconostoc mesenteroides</i> .....	Cienkowski; Van Tiegham	1878
<i>Lactobacillus casei</i> .....	von Freudenreich	1890
<i>Lactobacillus lactis</i> .....	Leichmann	1896
<i>Lactobacillus delbrueckii</i> .....	Leichmann; Lafar	1896
<i>Lactobacillus acidophilus</i> .....	Moro	1900
<i>Lactobacillus fermenti</i> .....	Beijerinck	1901
<i>Streptococcus faecalis</i> .....	Thiercelin	1902
<i>Lactobacillus buchneri</i> .....	Henneberg	1903
<i>Streptococcus salivarius</i> .....	Andrewes-Horder	1906
<i>Leuconostoc dextranicum</i> .....	Beijerinck	1912
<i>Lactobacillus plantarum</i> .....	Orla-Jensen	1919
<i>Lactobacillus pentoaceticus</i> .....	Fred-Peterson-Davenport	1919
<i>Leuconostoc citrovorum</i> .....	Hammer	1920
<i>Lactobacillus arabinosus</i> .....	Fred-Peterson-Davenport	1921
<i>Lactobacillus pentosus</i> .....	Fred-Peterson-Davenport	1921
<i>Lactobacillus lycopersici</i> .....	Mickle	1924

<sup>1</sup> Data taken from Bergey *et al.* (6).

TABLE 3. HENNEBERG'S<sup>1</sup> MEDIA FOR CULTURING LACTIC ACID BACTERIA

CONSTITUENT	MEDIUM		
	1	2	3
	%	%	%
Glucose.....	5.0	5.0	5.0
Peptone.....	1-5	1.0	1.0
Yeast infusion.....		1.0	
Prune infusion.....			1.0
KH <sub>2</sub> PO <sub>4</sub> .....	0.8		
MgSO <sub>4</sub> .....	0.1		

<sup>1</sup> Henneberg (55).

and Mendel (114) in 1913, it was not until 1926 that thiamine (63), 1933 that riboflavin (77) and 1940 that pantothenic acid (180) were available in crystalline form.

A most notable program of research on the nutrition of lactic acid bacteria was inaugurated in 1919 by Fred and Peterson (41-43, 60, 117, 123-132, 144, 165) at the University of Wisconsin. Their collaborators during succeeding years were Bohonos, Hutchings, Snell, Strong, Tatum, Wood, Woolley, Wright and other investigators who also have become eminent in this field. Although for more than a decade these

workers were concerned, primarily, with the fermentative products and processes of lactic acid and other bacteria cultured on yeast extract or cereal grain infusions, they identified and named *Lactobacillus pentoaceticus* (43), *Lactobacillus arabinosus* (42) and *Lactobacillus pentosus* (42).

In 1937 Snell, Tatum and Peterson (161) investigated the growth of *Lactobacillus delbrueckii* on a basal medium essentially the same as that employed in 1923 by Speakman (163) of the University of Toronto in his studies on the fermentation of glucose and other sugars by *Bacillus granulobacter pectinovorum*. The composition of Speakman's medium is given in table 4 and that of Snell *et al.* is shown as *medium 1* in table 5. Growth supplements employed by the latter workers included acid-hydrolyzed casein, tryptophan and an extract of potato or liver. The same year Snell, Strong and Peterson (159) made extensive studies on the nutrition of thirteen species of lactic acid bacteria. The basal medium (*medium 2*, table 5) was the same as *medium 1* except for the omission of the casein hydrolyzate and the addition of sodium acetate, cystine, riboflavin, and a supplement of liver, yeast or whey extract.

TABLE 4. BASAL MEDIUM USED BY SPEAKMAN<sup>1</sup> FOR FERMENTATION OF SUGARS BY BACILLUS GRANULOBACTER PECTINOVORUM

CONSTITUENT	PER CENT	GLUCOSE OR OTHER CARBOHYDRATE	PER CENT
Glucose or other carbohydrate....	3.0	MgSO <sub>4</sub> .....	0.02
Peptone.....	0.5	NaCl.....	0.001
KH <sub>2</sub> PO <sub>4</sub> .....	0.05	FeSO <sub>4</sub> .....	0.001
K <sub>2</sub> HPO <sub>4</sub> .....	0.05	MnSO <sub>4</sub> .....	0.001

<sup>1</sup> Speakman (163).

Riboflavin was incorporated into the medium because of Orla-Jensen's (113) report the preceding year that it was an essential growth factor for lactic acid bacteria.

The first thoroughly practicable microbiological procedure for the determination of any vitamin (riboflavin) was described by Snell and Strong (158) in 1939. The basal medium (*medium 3*, table 5) was the same as *medium 2* except for the omission of pantothenic acid and nicotinic acid, respectively, were reported by Pennington, Snell and Williams (121) in 1940 and by Snell and Wright (162) in 1941. The basal media were *media 4* and *5* in table 5. The basal medium utilized previously was altered markedly for use in the nicotinic acid assay by omitting peptone and adding tryptophan, adenine, guanine, uracil, riboflavin, pantothenic acid, pyridoxine, thiamine, an acid hydrolyzate of casein, and a concentrate of biotin. Riboflavin and pantothenic acid were determined with *Lactobacillus casei* and nicotinic acid with *Lactobacillus arabinosus* 17-5. These authors introduced pyridoxine and biotin into their basal medium because Möller (94, 95) had reported in 1938 and 1939 that these vitamins were essential or stimulatory for lactic acid bacteria. Pantothenic acid was added because the suggestion of Orla-Jensen (113) that it, in addition to riboflavin, was required by lactic acid bacteria had been corroborated by Snell, Strong and Peterson (160) in 1939.

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In 1946, Roberts and Snell (138) described a procedure considered to be suitable for the determination of five vitamins (biotin, folic acid, nicotinic acid, pantothenic acid and riboflavin) with *Lactobacillus casei* and a single basal medium. The medium

TABLE 5. BASAL MEDIA EMPLOYED BY SNELL ET AL. FOR VITAMIN ASSAYS  
(Values given as gm. %)

CONSTITUENT	MEDIUM					
	1 1937	2 1937	3 1939	4 1940	5 1941	6 1946
Glucose.....	1.0	1.0	1.0	1.0	1.0	2.0
Sodium acetate.....		0.6	0.6	0.6	0.6	2.0
Peptone.....	0.5	0.5	0.5	0.5		
Casein, enzymatic digest.....	5 <sup>1</sup>			0.2 <sup>1</sup>	0.5 <sup>1</sup>	2.0
K <sub>2</sub> HPO <sub>4</sub> .....	0.05	0.05	0.05	0.05	0.05	0.25
KH <sub>2</sub> PO <sub>4</sub> .....	0.05	0.05	0.05	0.05	0.05	0.25
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.02	0.02	0.02	0.02	0.02	0.08
NaCl.....	0.001	0.001	0.001	0.001	0.001	0.004
FeSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.001	0.001	0.001	0.001	0.001	0.004
MnSO <sub>4</sub> ·3H <sub>2</sub> O.....	0.001	0.001	0.001	0.001	0.001	0.016
Tryptophan.....	0.015	0.01			0.01	
Cystine.....		0.01	0.01	0.01	0.01	0.01
Adenine sulfate, mg. %.....					1	1
Guanine·HCl, mg. %.....					1	1
Uracil, mg. %.....					1	1
Riboflavin, γ %.....		10		10	20	50
Ca pantothenate, γ %.....					10	50
Nicotinic acid, γ %.....					20	50
Pyridoxine·HCl, γ %.....					20	50
Thiamine·HCl, γ %.....					10	50
p-Aminobenzoic acid, γ %.....						10
Biotin, γ %.....					Concen.	0.4
Folic acid, γ %.....						0.2
Supplement.....	S	S <sup>1</sup>	S <sup>2</sup>			

S, Liver or potato extract. S<sup>1</sup>, Liver, yeast or whey extract. S<sup>2</sup>, Yeast extract.

<sup>1</sup> Acid hydrolyzate.

- Col. 1. Snell, E. E., E. L. Tatum, and W. H. Peterson. *J. Bact.* 33: 207, 1937. Nutrition of *L. delbrueckii*.  
 Col. 2. Snell, E. E., F. M. Strong and W. H. Peterson. *Biochem. J.* 31: 1789, 1937. Nutrition of 13 lactic acid bacteria,  
 Col. 3. Snell, E. E. and F. M. Strong. *Ind. Eng. Chem., An. Ed.* 11: 346, 1939. Determination of riboflavin with *L. casei*.  
 Col. 4. Pennington, D., E. E. Snell and R. J. Williams. *J. Biol. Chem.* 135: 213, 1940. Determination of pantothenic acid with *L. casei*.  
 Col. 5. Snell, E. E. and L. D. Wright. *J. Biol. Chem.* 130: 675, 1941. Determination of nicotinic acid with *L. arabinosus* 17-5.  
 Col. 6. Roberts, E. C. and E. E. Snell. *J. Biol. Chem.* 163: 499, 1946. Determination of riboflavin and folic acid and standard curves for nicotinic acid, pantothenic acid and biotin with *L. casei*.

(medium 6, table 5) was identical in composition with that (medium 5, table 5) employed for the determination of nicotinic acid with *L. arabinosus* 17-5 except that an enzymatic digest of casein was substituted for acid-hydrolyzed casein, tryptophan was omitted, crystalline biotin was substituted for a biotin concentrate, and p-aminobenzoic acid and crystalline folic acid were added. The most drastic changes were who also hav.

marked increases in the concentration of glucose, sodium acetate, casein digest, buffer and other salts, and vitamins.<sup>3</sup>

A summary of the data on the requirements of 13 lactic acid bacteria<sup>4</sup> for 8 vitamins taken from recent publications by Shankman *et al.* (148) and by Snell (157) is given in table 6. Although the 6 organisms investigated in both laboratories were shown to have nearly the same vitamin requirements, from 1 to 2 organisms were found by Snell, but not by Shankman *et al.*, to require *p*-aminobenzoic acid, biotin, folic acid, thiamine and pyridoxine. A possible explanation for this difference is that the basal medium employed by Shankman *et al.* contained more nutrients, many at higher concentrations than other media utilized previously in studying the nutritional

TABLE 6. VITAMINS REQUIRED BY LACTIC ACID BACTERIA<sup>1</sup>

ORGANISM	<i>p</i> -AMINO-BENZOIC ACID	BIOTIN	FOLIC ACID	NICO-TINIC ACID	PANTO-THENIC ACID	RIBO-FLAVIN	THIA-MINE	PYRI-DOXINE
<i>L. arabinosus</i> 17-5 <sup>2</sup> .....	(X)	X		X	X			(X)
<i>L. brassicae</i> .....		X		X	X		X	
<i>L. buchneri</i> .....		X		X	X	X		
<i>L. casei</i> <sup>2</sup> .....		X	(X)	X	X	X		X
<i>L. delbrueckii</i> LD-5 <sup>2</sup> .....		X	(X)	X	X	X		X
<i>L. fermenti</i> 36 <sup>2</sup> .....		(X)		X	X			
<i>L. gayonii</i> .....				X	X			
<i>L. lycopersici</i> .....		X		X	X	X	X	
<i>L. manni-topoeus</i> .....		X		X	X		X	
<i>L. mesenteroides</i> P-60 <sup>2</sup> .....	(X)	X		X	X		(X)	(X)
<i>L. pentoaceticus</i> .....			X	X	X		X	
<i>L. pentosus</i> .....		X		X	X			
<i>S. fecalis</i> R.....		X	X	X	X			
No. vitamins essential.....	0	10	2	13	13	4	4	2

<sup>1</sup> Data taken from Shankman *et al.* (148) and Snell (157).

<sup>2</sup> Organism studied by Shankman *et al.* and Snell. All others studied only by Shankman *et al.*

(X) Vitamin considered essential by Snell only.

requirements of lactic acid bacteria. As shown in table 7 this basal medium contained 21 amino acids, 12 vitamins, 3 buffer and 5 other salts, 3 purines, 1 pyrimidine and 1 carbohydrate. It seems of particular interest that on this relatively complete medium, *p*-aminobenzoic acid was not required for the growth of any of the 13 organ-

<sup>3</sup> Microbiological procedures for the determination of riboflavin, pantothenic acid, biotin, nicotinic acid, pyridoxin, inositol, thiamin, and folic acid with *L. casei*, *Saccharomyces cerevisiae*, *L. arabinosus* 17-5, and *S. faecalis* (lactis R) were described in 1941 by Williams and co-workers (179). The content of these vitamins in various tissues of the rat, mouse, cow, and pig, and in milk, urine, and commercial animal rations was determined by these investigators.

<sup>4</sup> Excellent reviews on the nutrition of lactic acid and other bacteria have been presented by Burrows (14), Clifton (21), Henneberg (55), Kluyver (67), Knight (71, 72), Koser and Sanders (76), McIlwain (91), Orla-Jensen (111), Peskett (95), Peterson and Peterson (133), Snell (154, 156, 157), Stephenson (164) and Werkmann and Wood (176).

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isms listed in table 6 whereas folic acid was essential for only 2 organisms (pento-aceticus and fecalis R) and pyridoxine for only one (casei).<sup>5</sup>

Many types of biological materials have been assayed for vitamins by microbiological methods since 1939 and the data obtained have been of inestimable value in assessing the nutritional quality of rations and foods. It is recognized, however, that many of the data have only relative significance and that there are inherent difficulties which must be overcome before microbiological assays for vitamins in foods can be made with the degree of precision and accuracy attained by the best chemical methods in the analysis of natural products for some biological constituents. It may be mentioned in this connection that the vitamins for which microbiological

TABLE 7. BASAL MEDIUM EMPLOYED BY DUNN ET AL. IN STUDY OF LACTIC ACID BACTERIA NUTRITION<sup>1</sup>

CONSTITUENT		CONSTITUENT		CONSTITUENT	
	mg. %		mg. %		mg. %
DL-Alanine.....	66.7	DL-Serine.....	66.7	MnSO <sub>4</sub> ·4H <sub>2</sub> O.....	1
Asparagine, natural....	66.7	DL-Threonine.....	66.7	NaCl.....	35
L-Arginine·HCl.....	66.7	DL-Tryptophan.....	66.7	Thiamine·HCl.....	0.1
L-Cysteine·HCl.....	66.7	L-Tyrosine.....	66.7	Pyridoxine.....	0.16
L-Glutamic acid.....	66.7	DL-Valine.....	66.7	Pyridoxamine·2HCl....	0.01
Glycine.....	66.7	Adenine sulfate·2H <sub>2</sub> O..	1.4	Pyridoxal·HCl.....	0.01
L-Histidine·HCl·H <sub>2</sub> O...	66.7	Guanine·HCl·2H <sub>2</sub> O....	1.4	DL-Ca pantothenate....	0.2
L-Hydroxyproline.....	66.7	Uracil.....	1.2	Riboflavin.....	0.2
DL-Isoleucine.....	66.7	Xanthine.....	1.2	Nicotinic acid.....	0.2
L-Leucine.....	66.7	Glucose (gm.).....	2.0	Biotin.....	0.0005
DL-Lysine·HCl.....	66.7	Sodium acetate (gm.)..	1.2	p-Aminobenzoic acid...	0.01
DL-Methionine.....	66.7	NH <sub>4</sub> Cl (gm.).....	0.6	Folic acid (conc.).....	0.0005
DL-Norleucine.....	66.7	KH <sub>2</sub> PO <sub>4</sub> .....	50	Choline chloride.....	1
DL-Norvaline.....	66.7	K <sub>2</sub> HPO <sub>4</sub> .....	50	Inositol.....	2.5
DL-Phenylalanine.....	66.7	MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	20		
L-Proline.....	66.7	FeSO <sub>4</sub> ·7H <sub>2</sub> O.....	1		

<sup>1</sup> Dunn (34).

procedures are recommended by the Association of Vitamin Chemists (3) in their 1947 book entitled, *Methods of Vitamin Assay*, include only riboflavin, nicotinic acid and thiamine.<sup>6</sup>

It should be apparent from the foregoing discussion that the development in 1943 of microbiological methods for the determination of amino acids was the logical outcome of the extensive earlier studies on bacteriological media, bacterial metabolism, vitamins and amino acids. Various strains of lactic acid bacteria had been isolated and were readily maintained in pure culture. Reasonably well-defined basal media

<sup>6</sup> That *L. delbrueckii* LD-5 is the same as *L. casei* has been reported by Rogosa (139) and

<sup>5</sup> Dunn *et al.* (34).

<sup>6</sup> Microbiological procedures for the determination of thiamin, riboflavin, nicotinic acid, pantothenic acid, inositol, biotin, and folic acid, have been described by various workers (Hutchings, *et al.* 1943). *Methods of the Vitamins* by Dunn and Satterfield (23) who also have

which supported good growth of these organisms were available. Many of the B-complex vitamins had been isolated in crystalline form. Microbiological techniques, applicable to the determination of amino acids, had been developed for vitamin-assay purposes. Each of the 19 amino acids known to occur in proteins was obtainable in purified form either as the natural L-antipode or the synthetic DL-mixture.

Considerable time elapsed, however, after it was known that bacteria utilize amino acids before it was discovered that they could be employed to determine amino acids. More than 50 years ago aspartic acid and asparagine were added to basal media to stimulate the growth of microorganisms. In 1893 Uschinsky (172) cultivated diphtheria bacilli, and in 1911 Lowenstein and Pick (88) grew tubercle bacilli, on synthetic media containing aspartic acid. This practice<sup>7</sup> has been followed by other workers (2, 22, 65, 135, 143, 169) interested in growing the tubercle bacillus. Since 1922 basal media containing up to 19 amino acids have been employed in nutritional studies of numerous types of bacteria including *Corynebacterium* (97, 98, 100-103), *Salmonella* (15, 39, 87), *Clostridium* (20, 37, 38, 40, 68, 104, 105, 168, 183), *Streptococcus* (66, 89, 90, 96, 185), *Staphylococcus* (46, 69, 70, 177), *Brucella* (74), *Pasteurella* (24), *Escherichia* (36, 141) and other genera (10, 47, 118, 174).

As shown in table 8 it has been demonstrated, following the pioneer investigations of Koser and Rettger (75) in 1919, that *Eberthella typhosa* (119), *Corynebacterium diphtheriae* (97, 98, 100), *Clostridium sporogenes* (40), *Bacillus anthracis* (47), *Streptococcus hemolyticus* (90, 185), *Streptococcus faecalis* (145) and other organisms can be cultivated successfully on synthetic media. It was shown, furthermore, that from 1 to 6 amino acids were essential for the growth of each of these strains. Advantage has been taken of the assay potentialities of *Proteus morganii* by Pelczar and Porter (119, 120) in their microbiological method for the determination of pantothenic acid and, probably, it would have been possible to devise comparable procedures for the determination of tryptophan and several other amino acids. As shown in figure 1, the standard curves which Müeller (99) obtained in 1935 denoting the growth of diphtheria bacilli at different levels of cystine, methionine, glutamic acid or tryptophan resemble those used a decade later for the determination of these amino acids.

Orla-Jensen (113) found from his classical experiments reported in 1936 that *L. casei*, *L. helveticus* and other Lactobacilli could be cultured in a synthetic medium containing 17 amino acids provided a supplement of whey was added equivalent to one fourth of the total nitrogen in the complete medium. The growth response of these organisms was measured in terms of titratable acid produced. The next year Wood, Anderson, and Werkman (181) at Iowa State College cultivated *L. delbrueckii* and 5 other lactic acid bacteria in a basal medium containing in addition to 17 amino acids glucose, sodium acetate, ammonium sulfate and other inorganic salts, thiamine, riboflavin, and yeast extract. In 1939, Möller (95) grew *L. plantarum* on an analogous basal medium. Highly significant studies on the nutrition of *L. buchneri*, *L. lycopersici*, and *L. mannilopoeus* were reported by Wood, Geiger and Werkman (182) in 1940. These organisms were cultured successfully in a nearly-synthetic amino acid-containing basal medium and the standard curves shown in figure 2.

<sup>7</sup> The literature prior to 1930 on use of synthetic media for the growth of bacteria has been reviewed by Mayer (92). It should be noted that these same amino acids were also used at the same time by Landy (175) for the growth of *L. casei* and by Pollack (176) for the growth of *L. helveticus*.

*L. casei* and by Pollack



TABLE 8. SYNTHETIC MEDIA USED TO GROW BACTERIA (1919-1941)

CONSTITUENT	KOSER AND RETTGER 1919	FILDES <i>et al.</i> 1933	MUELLER 1934	FILDES AND RICHARDSON 1935	GLADSTONE 1939	MCILWAIN 1940	WOOLLEY AND HUTCHINGS 1940	KOSER <i>et al.</i> 1941	SCHEUHAN <i>et al.</i> 1941
Glucose.....	x	x			x	x	x	x	x
Liebig's extract.....			x						
Ash from hay.....				x					
Sodium citrate.....		x							x
Iron salt.....			x		x	x			
NH <sub>4</sub> Cl.....		x				x			
NaCl.....	x	x	x				x	x	x
MgSO <sub>4</sub> .....	x	x	x		x	x	x	x	x
CaCl <sub>2</sub> .....	x		x					x	
KH <sub>2</sub> PO <sub>4</sub> .....	x	x	x	x	x	x	x		x
K <sub>2</sub> HPO <sub>4</sub> .....	x	x	x	x	x	x		x	x
Alanine.....		x		x	x	x	x	x	x
Arginine.....		x		x	x	x	x	x	x
Aspartic acid.....	x	x		x	x	x	x		x
Cystine.....		x	x	x	x	x	x	x	x
Glutamic acid.....	x	x	x		x	x	x	x	x
Glycine.....	x	x	x	x	x	x	x	x	x
Histidine.....	x	x	x	x	x	x	x	x	x
Hydroxyproline.....					x	x	x	x	x
Isoleucine.....				x	x	x	x		x
Leucine.....		x		x	x	x	x	x	x
Lysine.....	x	x		x	x	x	x	x	x
Methionine.....			x	x	x	x	x	x	x
Phenylalanine.....		x	x	x	x	x	x	x	x
Proline.....		x			x	x	x	x	x
Serine.....				x		x	x	x	x
Threonine.....					x	x	x	x	x
Tryptophan.....	x	x	x	x	x	x	x	x	x
Tyrosine.....	x	x		x	x	x	x	x	x
Valine.....	x	x	x	x	x	x	x	x	x

x—signifies amino acid essential for growth of organism.

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- KOSER, S. A. AND L. F. RETTGER. *J. Infect. Dis.* 24: 301, 1919. Medium contained taurine, creatine and allantoin; 24 non-lactic acid organisms were tested.
- FILDES, P., G. P. GLADSTONE, AND B. C. J. G. KNIGHT. *Brit. J. Exper. Path.* 14: 189, 1933. Organism tested was *Eberthella typhosa*.
- MUELLER, J. H. *Proc. Soc. Exper. Biol. & Med.* 32: 318, 1934, and *J. Bact.* 39: 515, 1935; 30: 513, 1935. Supplements tested were thiamine, pantothenic acid and extracts of liver, muscle and heart. Diphtheria bacillus was the organism tested.
- FILDES, P. AND G. M. RICHARDSON. *Brit. J. Exper. Path.* 16: 326, 1935. Organism tested was *Clostridium sporogenes*.
- GLADSTONE, G. P. *Brit. J. Exper. Path.* 20: 189, 1939. Organism tested was *Bacillus anthracis*.
- MCILWAIN, H. *Brit. J. Exper. Path.* 21: 25, 1940. Supplements tested were ornithine, citrulline,  $\beta$ -hydroxyglutamic acid, glucosamine, inositol, thiochrome, pyridoxine, pantothenic acid,  $\beta$ -alanine, riboflavin, betaine, and hydrolyzates of casein, yeast and egg albumin. Organism tested was *Streptococcus hemolyticus*.
- WOOLLEY, D. W. AND B. L. HUTCHINGS. *J. Bact.* 39: 287, 1940. Basal medium contained Na<sub>2</sub>SO<sub>4</sub>, reduced iron, riboflavin, pyridoxine, and a pantothenic acid concentrate. Organism tested was *Streptococcus symogenes* strain H-6905 (Lancefield Group D).
- KOSER, S. A., B. B. BRESLOV, AND A. DORFMAN. *J. Infect. Dis.* 69: 114, 1941. Supplements tested were nicotinamide, thiamine, cocarboxylase,  $\beta$ -alanine, Ca pantothenate, pyridoxine, riboflavin, inositol, glutamine, adenine, biotin, biotin concentrate, biotin concentrate and traces of iron, manganese, copper and calcium salts. Organisms tested were *Brucella abortus*, *Brucella melitensis*, *Brucella abortus* and *Brucella abortus* (the Brucella group).
- SCHEUHAN, J. H. *J. Infect. Dis.* 69: 81, 1941. Basal medium contained riboflavin, pantothenic acid and biotin. Organism tested was *Streptococcus faecalis* (Lancefield Type D).

tained for 12 amino acids. Although relatively little acid was produced, owing primarily to the low concentration of amino acids in the medium, it is evident that probably it would not have been difficult to adapt the procedures of Wood *et al.* to the microbiological determination of amino acids.

As was stated earlier in this discussion the *L. arabinosus*-microbiological assay procedures of Kuiken *et al.* (79, 80) for the determination of isoleucine, leucine and

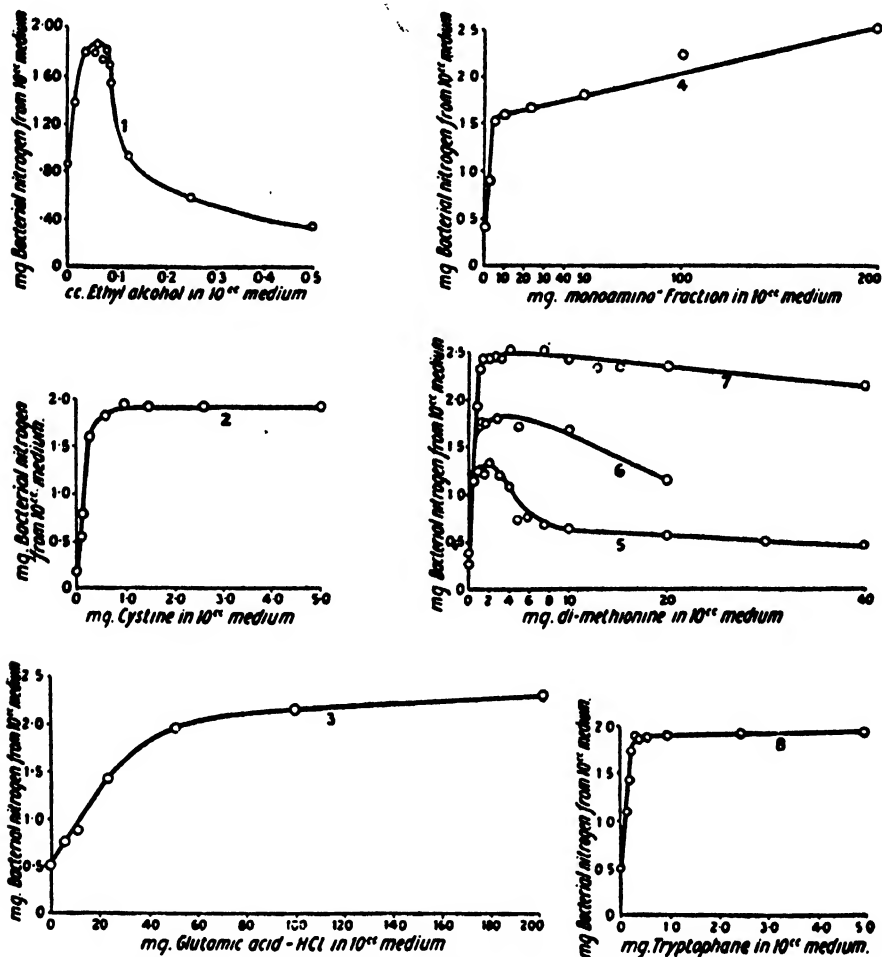


Fig. 1. STANDARD CURVES for 4 amino acids based on acid produced by *C. diptheriae*. (MUELLER, J. H. *J. Bact.* 29: 522, 1935).

valine were the first to be reported. The composition of the basal media employed in 1943 by Greene and Black (49) for the determination of tryptophan with *L. arabinosus* and by Shankman *et al.* (150, 151) for the determination of several amino acids with *L. arabinosus* and *L. casei* is given in table 9. It may be noted that these basal media were patterned after those formulated about the same time by Landy and Dicken (81) for the determination of six vitamins with *L. casei* and by Pollack

and Linder (134), Bohonos *et al.* (7), Hutchings and Peterson (62), Shankman (147), Smith (152) and Gaines and Stahley (45) for studies on the nutrition of *L. arabinosus*, *L. casei*, *L. pentosus*, *L. delbreuckii*, *Leuconostoc mesenteroides*, and *S. fecalis* R (*lactis*).

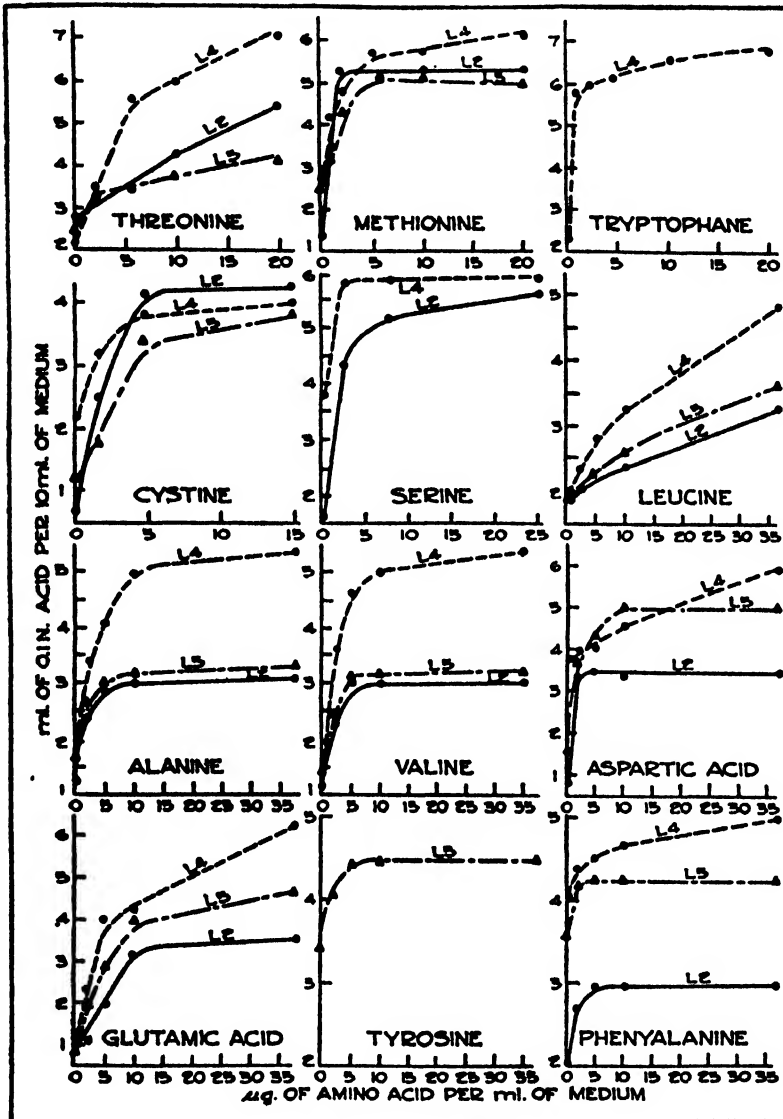


Fig. 2. STANDARD CURVES for 12 amino acids based on acid produced by *L. buchneri*, *L. lycopersici* and *L. manitopoeus* (WOOD, GEIGER AND WERKMAN. *Ia. State Coll. J. Sci.* 14: 367, 1939-40).

As shown in table 9, changes made in the basal media during the past five years include the addition of ammonium sulfate, ammonium chloride, sodium citrate, succinic acid, inositol, choline, folic acid, pyridoxamine, pyridoxal, xanthine, as-

paragine, glycine, hydroxyproline, norleucine and norvaline. Most workers have increased the concentration of glucose from 1 to 2 per cent, and in a few instances to 3 (26, 28) and even 4 per cent (173), to promote greater acid production and, ostensibly, to increase the convenience and accuracy of the titrations. The concentration of sodium acetate has been increased from 0.6 per cent to as much as 2.4 per cent (173) to facilitate greater acid production without significant decrease in  $pH$ . This advantage is offset somewhat by the increased difficulty with which the color change of the bromthymol blue indicator is detected in the visual titration procedure. Sodium citrate has been substituted for sodium acetate (137, 142) and both buffer salts have been introduced into some basal media (54) because of "the superiority of citrate over acetate in promoting growth and acid production (of *S. fecalis*) . . ." and "its (sodium acetate's) demonstrated growth-stimulating activity for several lactic acid bacteria. . . ."  $KH_2PO_4$  was omitted and the concentration of the other usual salts was increased from 2- to 10-fold in the 1948 medium of Henderson and Snell (54). The concentration of the vitamins has been increased 10-fold, or even more, by most of the later investigators. The concentration of adenine, guanine and uracil has also been increased considerably by many workers. In general, nearly all of the basal media have contained about the same concentrations of amino acids as those employed initially by Kuiken *et al.* (79).

It may be noted from table 10 that all of the 19 amino acids, except hydroxyproline, which occur commonly in proteins, have been determined by microbiological methods in various natural materials. Each of the 18 amino acids has been determined by from 1 to 4 of the 5 organisms which have been employed commonly for this purpose. Three amino acids have been determined with *L. fermenti* 36, 10 each with *L. arabinosus* 17-5 and *L. casei*,<sup>5</sup> 14 with *S. fecalis* R and 18 with *Leuconostoc mesenteroides* P-60. That no assay procedure has been found for hydroxyproline is explained by the failure to discover any organism for which this amino acid is a growth-essential. Its uniqueness in this respect may indicate that hydroxyproline is synthesized by the lactic acid bacteria which have been studied or that it is not an essential metabolite.

The extreme fastidiousness of *L. mesenteroides* P-60 for amino acids was observed in 1944 by Dunn *et al.* (35) who obtained the standard curves shown in figure 3. Although hydroxyproline, norleucine and norvaline were found to be inactive, 18 other amino acids appeared to be growth promoters. Later workers have taken advantage of the assay potentialities of *L. mesenteroides* P-60 in determining all but one of the 18 amino acids for which standard curves have been reported. As shown in table 11, food proteins, corn steep liquor, urine, enzymes, viruses, bacterial toxins and other materials have been assayed for these amino acids with this organism in the laboratories of Barton-Wright (4, 5), Buehler (11), Cardinal and Hedrick (19), Dunn (29-32, 149), Heller and Kirch (53), Hier (57), Jones (61), Knight (73), Sauberlich and Baumann (142), Snell (50, 54), and Velick and Ronzoni (173). The experiments reported by Dunn *et al.* (34) in 1947 strengthened the view that assays for 15 amino acids might be made satisfactorily with *L. mesenteroides* P-60. It seemed doubtful, however, that alanine, proline or serine could be determined accurately, especially in foods and other complex mixtures of nutrients, since none of

TABLE 9. COMPOSITION OF BASAL MEDIA USED TO DETERMINE AMINO ACIDS WITH LACTIC ACID BACTERIA  
(Values given as percentage)

CONSTITUENT	1942			1943								1944				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Glucose, gm.....	1.0	2.0	1.0	2.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0	1.0	1.0	1.75	2.0	2.0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , gm.....														0.3	0.6	2.0
NaAc, gm.....	0.6 <sup>7</sup>	1.2	0.6	0.6	0.6	0.6	0.6	0.6 <sup>8</sup>	0.6	0.6	1.45	0.6	0.6	0.875	0.6	2.0
NNH <sub>4</sub> Cl, gm.....																
KKH <sub>2</sub> PO <sub>4</sub> , gm.....	0.05	0.1	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.1	0.05	0.05	0.05
K <sub>2</sub> HPO <sub>4</sub> , gm.....	0.05	0.1	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.1	0.05	0.05	0.05
MgSO <sub>4</sub> ·7H <sub>2</sub> O, gm.....	0.02	0.04	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
NaCl, gm.....	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
FeSO <sub>4</sub> ·7H <sub>2</sub> O, gm.....	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
MnSO <sub>4</sub> ·4H <sub>2</sub> O, gm.....	0.00067	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Inositol, γ.....		500											250			
Choline chloride, γ.....													250			
Thiamine·HCl, γ.....	10	20			10	10	10	10	10	10	10	10	10	40		20
Pyridoxine·HCl, γ.....	40	20		20	10	10	10	10	10	20	10	10	30	40	10	20
Ca-Pantothenate, γ.....	20	20	20	20	10	10	10	10		20	10	10	10	40	10	20
p-Aminobenzoic acid, γ.....					1	S	1				0.05		30	40	10	10
Riboflavin, γ.....	20	20	10	20	20	20	20	20	20	20	20	20	10	40	10	40
Nicotinic acid, γ.....	20	20	20	20	20	20	20	10		20	40	20	10	40	10	20
Biotin, γ.....	0.5	0.04		0.01	0.04	0.004	0.04	0.1		0.01	0.04	0.04	0.02	0.04	0.04	0.02
Folic acid, γ.....	0.05	0.5			S			0.005		1.0			1		0.025	
Adenine sulfate, mg.....	0.5	1		2	1	1	1	1	1	2	1	1	1	0.4	1	1
Guanine·HCl, mg.....	0.5	1			1	1	1	1	1		1	1	1	0.4	1	1
Uracil, mg.....	0.5	1			1	1	1	1	1				1	0.4		
Xanthine, mg.....	0.5	1						1					1			
Alanine, mg.....		1*		20*	20*	20	20*			10*	20*		20*	8.3*		20*
Aspartic acid, mg.....		1		20*	40	40	40			10*	40*		40	8.3*		40
Asparagine, mg.....		1														
Arginine·HCl, mg.....	25	1		20	5	20	5	8		10	20		10	8.3		5

CONSTITUENT	1044										1045									
	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32				
Glucose, gm.....	1.0	2.0	2.0	1.0	1.66	2.0	1.0	2.0	2.0	2.0	1.75	1.0	2	1	2	1				
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , gm.....											0.3	0.3								
NaAc, gm.....	0.6	1.2	1.2	0.6	1.3		1.07	1.2	0.725	0.6	0.875	0.6	17	0.67	1.2	0.6				
NH <sub>4</sub> Cl, gm.....		0.6	0.6												0.6					
KH <sub>2</sub> PO <sub>4</sub> , gm.....	0.05	0.05	0.05	0.05	0.03	0.45	0.05	0.054	0.05	0.05	0.05	0.1	0.05	0.05	0.05	0.05				
K <sub>2</sub> HPO <sub>4</sub> , gm.....	0.05	0.05	0.05	0.05	0.03		0.05	0.054	0.05	0.05	0.05	0.1	0.05	0.05	0.05	0.05				
MgSO <sub>4</sub> ·7H <sub>2</sub> O, gm.....	0.02	0.02	0.02	0.02	0.013	0.004	0.02	0.0216	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02				

CONSTITUENT	1044										1045									
	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32				
Glucose, gm.....	1.0	2.0	2.0	1.0	1.66	2.0	1.0	2.0	2.0	2.0	1.75	1.0	2	1	2	1				
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , gm.....											0.3	0.3								
NaAc, gm.....	0.6	1.2	1.2	0.6	1.3		1.07	1.2	0.725	0.6	0.875	0.6	17	0.67	1.2	0.6				
NH <sub>4</sub> Cl, gm.....		0.6	0.6												0.6					
KH <sub>2</sub> PO <sub>4</sub> , gm.....	0.05	0.05	0.05	0.05	0.03	0.45	0.05	0.054	0.05	0.05	0.05	0.1	0.05	0.05	0.05	0.05				
K <sub>2</sub> HPO <sub>4</sub> , gm.....	0.05	0.05	0.05	0.05	0.03		0.05	0.054	0.05	0.05	0.05	0.1	0.05	0.05	0.05	0.05				
MgSO <sub>4</sub> ·7H <sub>2</sub> O, gm.....	0.02	0.02	0.02	0.02	0.013	0.004	0.02	0.0216	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02				

CONSTITUENT	1044										1045									
	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32				
Glucose, gm.....	1.0	2.0	2.0	1.0	1.66	2.0	1.0	2.0	2.0	2.0	1.75	1.0	2	1	2	1				
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , gm.....											0.3	0.3								
NaAc, gm.....	0.6	1.2	1.2	0.6	1.3		1.07	1.2	0.725	0.6	0.875	0.6	17	0.67	1.2	0.6				
NH <sub>4</sub> Cl, gm.....		0.6	0.6												0.6					
KH <sub>2</sub> PO <sub>4</sub> , gm.....	0.05	0.05	0.05	0.05	0.03	0.45	0.05	0.054	0.05	0.05	0.05	0.1	0.05	0.05	0.05	0.05				
K <sub>2</sub> HPO <sub>4</sub> , gm.....	0.05	0.05	0.05	0.05	0.03		0.05	0.054	0.05	0.05	0.05	0.1	0.05	0.05	0.05	0.05				
MgSO <sub>4</sub> ·7H <sub>2</sub> O, gm.....	0.02	0.02	0.02	0.02	0.013	0.004	0.02	0.0216	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02				

CONSTITUENT	1044										1045									
	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32				
Glucose, gm.....	1.0	2.0	2.0	1.0	1.66	2.0	1.0	2.0	2.0	2.0	1.75	1.0	2	1	2	1				
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , gm.....											0.3	0.3								
NaAc, gm.....	0.6	1.2	1.2	0.6	1.3		1.07	1.2	0.725	0.6	0.875	0.6	17	0.67	1.2	0.6				
NH <sub>4</sub> Cl, gm.....		0.6	0.6												0.6					
KH <sub>2</sub> PO <sub>4</sub> , gm.....	0.05	0.05	0.05	0.05	0.03	0.45	0.05	0.054	0.05	0.05	0.05	0.1	0.05	0.05	0.05	0.05				
K <sub>2</sub> HPO <sub>4</sub> , gm.....	0.05	0.05	0.05	0.05	0.03		0.05	0.054	0.05	0.05	0.05	0.1	0.05	0.05	0.05	0.05				
MgSO <sub>4</sub> ·7H <sub>2</sub> O, gm.....	0.02	0.02	0.02	0.02	0.013	0.004	0.02	0.0216	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02				

TABLE 9.—Continued

CONSTITUENT	1944					1945										
	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
NaCl, gm.....		0.001	0.001	0.001	0.00066					0.001	0.001	0.001	0.001	0.001	0.001	0.001
FeSO <sub>4</sub> ·7H <sub>2</sub> O, gm.....	0.001	0.001	0.001	0.001	0.00066		1.0		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
MnSO <sub>4</sub> ·4H <sub>2</sub> O, gm.....	0.001	0.001	0.001	0.001	0.00066		0.001	0.00108	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Inositol, γ.....												250				
Choline chloride, γ.....												250				
Thiamine·HCl, γ.....	100	100	100	100	13		50	40	10		40	50	100	100	100	20
Pyridoxine·HCl, γ.....	160	160	160	20	13		50	64	10	20*	40	1000	200	200	160	40 <sup>d</sup>
Ca-pantothenate, γ.....	200*	200	200	20	26		10	80	10	20	40	20	100	100	200	20
p-Aminobenzoic acid, γ.....	10	0.01	0.01	20	13		1	4	0.05		40	30	50	50	0.01	4
Riboflavin, γ.....	200	200	200	20	26		20	80	20	20	40	20	100	100	200	20
Nicotinic acid, γ.....	200	200	200	40	26		50	80	40	20	40	20	100	100	200	20
Biotin, γ.....	0.05	0.5	0.5	0.5	0.06		1	0.2	0.04	0.01	0.04	0.1	0.5	0.5	0.5	0.02
Folic acid, γ.....	0.4	0.2	0.2	S						0.1		1.0			0.2	0.2
Adenine sulfate, mg.....	10	1.2	1.2	5	0.66		1	1.2	0.5	2	0.4	1	0.5	5	1.2	1
Guanine·HCl, mg.....	10	1.2	1.2	5	0.66		1	1.2	0.5		0.4	1	0.5	5	1.2	1
Uracil, mg.....	10	1.2	1.2	5	0.66		1	1.2	0.5		0.4	1	0.5	5	1.2	1
Xanthine, mg.....											0.4	1	0.5	5		
Alanine, mg.....	20*	200*	200*	20*		11.9*			20*	20*	8.3*	20*		20*	200*	20*
Aspartic acid, mg.....				20*		17.7*			40*	20*	8.3*	40		20*		20*
Asparagine, mg.....	20	40	40	20					20	20	8.3	10	10	20	8	20
Arginine·HCl, mg.....	5	8	8	20				12	20	20	8.3	10	10	20	12	20
Cystine, mg.....	10	12	12	20	6.6	5.2	10		20	20	8.3	100*		20	15	20*
Glutamic acid, mg.....	†	15	15	20		6	†		† <sup>b</sup>	20*	8.3	10	20	20	10	20
Glycine, mg.....	10	10	10	20		10			20	20	8.3	10	20	20	20	20
Histidine·HCl·H <sub>2</sub> O, mg.....	5	2	2	20		5			20	20	8.3	10	20	20*	2	20
Hydroxyproline, mg.....	10	10	10	20		10.9				20	8.3	10	20	20	10	20
Isoleucine, mg.....	20*	15*	15*	20*					20*	20*	8.3*	20*	20*	20*	15*	20*
Leucine, mg.....	20	7.5	7.5	20		17.5*			20*	20*	8.3	10	20	20	7.5	20*
Lysine·HCl, mg.....	20*	16*	16*	20*		4.6			20	10	8.3	10	20	20	16*	10

	10*	4*	20*	7.5	20*	8.3*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*	20*</
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TABLE 9.—Continued

CONSTITUENT	1945							1946								
	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
Biotin, $\gamma$ .....	0.5	0.5	0.04	0.5	0.5	0.5	0.5	0.5	20	0.05	0.05	0.5	0.04	0.5	0.1	0.1
Folic acid, $\gamma$ .....	0.2	0.6		0.2	0.2	0.2	0.2	S				0.1	0.2	0.2	1.0	1.0
Adenine sulfate, mg.....	1.2	1.2	1	2.4	1.2	1.0	2.4	0.05	1	1	1	1.2	1	1.2	2	2
Guanine-HCl, mg.....	1.2	1.2	1	2.4	1.2	1.0	2.4	0.05	1	1	1	1.2	1	1.2	2	2
Uracil, mg.....	1.2	1.2	1	2.4	1.2	1.0	2.4	0.05	1	1	1	1.2	1	1.2	2	2
Xanthine, mg.....				2.4		2.4				1	1	1.2	1		2	2
Alanine, mg.....	400*	20*	20*	40*	266*	20*	100*	20*			20*	100*	20*	100*	40*	40*
Aspartic acid, mg.....											80*	80*	20*	80*	80	80
Asparagine, mg.....	80	20	40	20	53	40	50	20			5	25	20	25	60	20
Arginine-HCl, mg.....	16	20	5	16	11	5	40	20	20		10	10	20	10	20	20
Cystine, mg.....	24	27.5 (HCl)	10	20	16	5 <sup>p</sup>	50	20	20	20						
Glutamic acid, mg.....	30	20	40	24	20	40	60	20			40	50	20	50	200	200
Glycine, mg.....	20		10	20	13		50	20				10	20	10	20	20
Histidine-HCl·H <sub>2</sub> O, mg.....	4	20	5	9	3	5	9	20			5	10	20	10	20	20
Hydroxyproline, mg.....	20		10	20	13		50	20				20*	20*	20*	40*	40*
Isoleucine, mg.....	30*	20*	20*	40*	20*	20*	100*	20*			20*	20*	20*	20*	40*	40*
Leucine, mg.....	15	20	20*	20	10	20	50	20			10	10	20	10	20	20
Lysine-HCl, mg.....	32*	20	20	20	21*	20	50	20*			40*	25*	20*	25*	20	20
Methionine, mg.....	8*	20*	10*	20*	5*	10*	50*	20*			10*	10*	20*	10*	40*	40*
Norleucine, mg.....	20*		10*	20*	13*		50*	20*				10*	20*	10*	40*	40*
Norvaline, mg.....	20*			20*	13*		50*	20*			10*	10*	20*	10*	40*	40*
Phenylalanine, mg.....	12*	†	10*	20*	8*	10*	50*	20*			10*	10*	20*	10*	40*	40*
Proline, mg.....	5		10	20	3.3		50	20				20*	20*	10	20	20
Serine, mg.....	16*	30*	5*	20*	11*		50*	20*				10*	20*	10*	40*	40*
Threonine, mg.....	90*	20*	20*	20*	60*		50*	20*			20*	50*	20*	50*	40*	40*
Tryptophan, mg.....							8	20								
Tyrosine, mg.....	2	20	4	3.2	1.3	3.3	25	20	10	†	8*	10*	60*	10*	20	20
Valine, mg.....	6	20	4	10	4	3.3	100*	20*			4	10	20	10	20	20
Casein (acid hydrolyzate), gm.....	30*	20*	20*	40*	20*	20*		20*	0.5	0.6	20*	20*	20*	20*	40*	40*
Organism.....	M	C	A	F	M	A	F	A-C-F	A	A	A	M	F	M	M	F

CONSTITUENT	1046													
	49	50	51	52	53	54	55	56	57	58	59	60	61	62
Glucose, gm.....	2	2					2	2	2	2	1	2	2	2
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , gm.....														
NaAc, gm.....	2	2.5*	2	2	2.5*	2	2	2	0.3*	1.2	0.6	2*	2	1.2
NH <sub>4</sub> Cl, gm.....				0.3	0.3					0.6				0.6
KH <sub>2</sub> PO <sub>4</sub> , gm.....	0.05		0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05		0.05	0.05
K <sub>2</sub> HPO <sub>4</sub> , gm.....	0.05	0.5	0.05	0.05	0.5	0.05	0.05	0.05	0.05	0.05	0.05	0.25	0.05	0.05
MgSO <sub>4</sub> ·7H <sub>2</sub> O, gm.....	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
NaCl, gm.....	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
FeSO <sub>4</sub> ·7H <sub>2</sub> O, gm.....	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
MnSO <sub>4</sub> ·4H <sub>2</sub> O, gm.....	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Inositol, γ.....	250	250	250											
Choline chloride, γ.....	250	250	250											
Thiamine-HCl, γ.....	50	50	50	50	50	50	50	50	20	100		20	100	100
Pyridoxine-HCl, γ.....	500 <sup>b</sup>	500 <sup>a</sup>	500 <sup>b</sup>	100	100	100	100	100	40 <sup>1</sup>	200	20	120 <sup>k</sup>	20	160
Ca-pantothenate, γ.....	50*	50*	50*	50*	50*	50*	50*	50*	20	200	20	40	20	200*
p-Aminobenzoic acid, γ.....	50	50	50	10	10	10	10	10	0.1	0.01	20	1	10	0.01
Riboflavin, γ.....	50	50	50	50	50	50	50	50	50	200	20	20	40	200
Nicotinic acid, γ.....	100	100	100	100	100	100	100	100	100	200	40	60	20	200
Biotin, γ.....	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.5	0.05	0.04	0.02	0.5
Folic acid, γ.....	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.5	0.15	0.2	0.2	0.2	0.2
Adenine sulfate, mg.....	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.5	1.0	5	1.0	1.0	1.2
Guanine-HCl, mg.....	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.5	1.0	5	1.0	1.0	1.2
Uracil, mg.....	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.5	1.0	5	1.0	1.0	1.2
Xanthine, mg.....	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.5	1.0	5	1.0	1.0	1.2
Alanine, mg.....	20*	10*	20*	20*	10*	20*	1.0	1.0	0.5		4*	1.0		200*
Aspartic acid, mg.....						†			20*		12*			
Asparagine, mg.....	40	40	40	20	20	20			40*					40
Arginine-HCl, mg.....	5	5	10	5	5	10								8
Cystine, mg.....	10	20	20	10	20	20	10	10	20	10	20		20	12 <sup>p</sup>

Table 9.—Continued

CONSTITUENT	1946																1948			
	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65			
Glutamic acid, mg.....	40	40	40 <sup>a</sup>	40 <sup>a</sup>	40 <sup>a</sup>	40			40 <sup>a</sup>		42 <sup>a</sup>			15	40	40	40			
Glycine, mg.....		2	10	2	2	10		10	20		20			10	20	20	20			
Histidine·HCl·H <sub>2</sub> O, mg.....	5	5	10	5	5	10			20		2.7			2	20	20	20			
Hydroxyproline, mg.....	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>			20 <sup>a</sup>		1			10	20	20	20			
Isoleucine, mg.....	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>			20 <sup>a</sup>		2.5 <sup>a</sup>			15 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>			
Leucine, mg.....	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>			20 <sup>a</sup>		20 <sup>a</sup>			7.5	20	20	20			
Lysine·HCl, mg.....	20	20	20	20	20	20		10	20		15 <sup>a</sup>			16 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>			
Methionine, mg.....	20 <sup>a</sup>	10 <sup>a</sup>	10 <sup>a</sup>	10 <sup>a</sup>	10 <sup>a</sup>	20 <sup>a</sup>		20 <sup>a</sup>	†		5			4 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>			
Norleucine, mg.....											6 <sup>a</sup>			10 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>			
Norvaline, mg.....	10 <sup>a</sup>	10 <sup>a</sup>	10 <sup>a</sup>	10 <sup>a</sup>	10 <sup>a</sup>	10 <sup>a</sup>			20 <sup>a</sup>		8 <sup>a</sup>			6 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>			
Phenylalanine, mg.....			5	5	5	5		5	20		7			2.5	20	20	20			
Proline, mg.....		5 <sup>a</sup>	20 <sup>a</sup>	5 <sup>a</sup>	5 <sup>a</sup>	20 <sup>a</sup>		10 <sup>a</sup>	20 <sup>a</sup>		12 <sup>a</sup>			8 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>			
Serine, mg.....	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>			20 <sup>a</sup>		9 <sup>a</sup>			45 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>			
Threonine, mg.....	5 <sup>a</sup>	10 <sup>a</sup>	10 <sup>a</sup>	5 <sup>a</sup>	10 <sup>a</sup>	10 <sup>a</sup>	5 <sup>a</sup>	10 <sup>a</sup>	10	5	10			1.0	20	20	20			
Tryptophan, mg.....	5	10	10	5	10	10		10	20 <sup>a</sup>	10 <sup>a</sup>	6.5			3	20	20	20			
Tyrosine, mg.....	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>			20 <sup>a</sup>		12 <sup>a</sup>			15 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>	20 <sup>a</sup>			
Valine, mg.....	0.2 <sup>a</sup>	0.5 <sup>a</sup>	0.5 <sup>a</sup>	0.5 <sup>a</sup>	0.5 <sup>a</sup>	0.5 <sup>a</sup>	0.5	0.5 <sup>a</sup>	0.5 <sup>a</sup>	0.75 <sup>a</sup>		0.5								
Casein (acid hydrol.), gm.....	A	F	M	A	F	M	A	M	F	M	A	C	A	M	A	F	F			
Organism.....															C-F					

CONSTITUENT	1947												1948			
	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	
Glucose, gm.....	2	2	2	2	1	1	2	2	2	2	2	2	2	2	2	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , gm.....																
NaAc, gm.....	0.3 <sup>t</sup>	2	1.2	1.2	0.6	0.6	0.3 <sup>t</sup>	0.3 <sup>t</sup>	2	2		1.2	2	0.1 <sup>f</sup>	1.2	
NH <sub>4</sub> Cl, gm.....			0.6	0.6					0.3			0.6	2	0.3	0.4	
KH <sub>2</sub> PO <sub>4</sub> , gm.....	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05			0.05	0.05		0.055	

[illegible]

TABLE 9—Continued

CONSTITUENT	1947											1948			
	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
Proline, mg.....	20		66.7	5	7	7	20		10*	10*	10*	3-3		10	4.6
Serine, mg.....	7.5*		66.7*	16*	12*	12*	20*		20*			10.7*		20*	9.2*
Threonine, mg.....	20*		66.7*	90*	9*	9*	20*		20*			60*	20*	20*	9.2*
Tryptophan, mg.....	20	5	66.7*	2	10	10	20*	†	10*	10*	10*	1.3	4	10	4.6*
Tyrosine, mg.....	20*		66.7	6	6.5	6.5	20*	10	10			4	4	10	9.2
Valine, mg.....	20*		66.7*	30*	12*	12*	20*		20*	0.5 <sup>r</sup>	0.5 <sup>r</sup>	20*	20*	20*	34*
Casein (acid hydrolyzate), gm.....		0.5 <sup>r</sup>						0.5 <sup>r</sup>	B'	B'	B'		A	A-C-D	A
Organism.....	F	F	B	M	M	F	F	F	B'	B'	B'	M		F-Fer	M

CONSTITUENT	1948														
	81	82	83	84	85	86	87	88	89	90	91	92			
Glucose, gm.....	2	1	1	2	4	1	2	2	1	1	1	2			
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , gm.....	0.3			0.6		0.3				0.3			1.2		
NaAc, gm.....	2.0	0.6	0.6	1.2	2.4	0.6	1.2	0.6	0.6 <sup>f</sup>	0.6	0.6	0.6	0.6		
NH <sub>4</sub> Cl, gm.....					1.2		0.6	0.3					0.6		
KH <sub>2</sub> PO <sub>4</sub> , gm.....	0.25	0.05	0.05	0.05	0.1	0.1	0.1	0.1	0.05	0.1	0.05	0.05	0.05		
K <sub>2</sub> HPO <sub>4</sub> , gm.....	0.25	0.05	0.05	0.05	0.1	0.1	0.1	0.1	0.05	0.1	0.05	0.05	0.05		
MgSO <sub>4</sub> ·7H <sub>2</sub> O, gm.....	0.08	0.02	0.02	0.02	0.04	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02		
NaCl, gm.....	0.004	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
FeSO <sub>4</sub> ·7H <sub>2</sub> O, gm.....	0.004	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
MnSO <sub>4</sub> ·4H <sub>2</sub> O, gm.....	0.016	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001		
Inositol, γ.....						250		250		250					
Choline chloride, γ.....		20				250		250		250					
Thiamine·HCl, γ.....	100			100	200	10	100	50	100	10	20	100			
Pyridoxine·HCl, γ.....	100	40 <sup>d</sup>	20	160	320	30	160	160	20	30	200	160			

Organism.....	F	F	F	M	M	A	M	M	M	M	M	C	D	M
Ca-Pantothenate, $\gamma$ .....	100	20	20	200	400	10	200	20	20	10	20	10	20	200
p-Aminobenzoic acid, $\gamma$ .....	5	4		0.01	0.02	30	0.1	0.1	20	30	4	30	4	0.01
Riboflavin, $\gamma$ .....	100	20	20	200	400	10	200	20	20	10	20	10	20	200
Nicotinic acid, $\gamma$ .....	100	20	20	200	400	10	200	20	40	10	20	10	20	200
Biotin, $\gamma$ .....	0.5	0.02	0.01	0.5	1.0	0.02	0.5	0.1	0.5	0.02	0.02	0.02	0.02	0.5
Folic acid, $\gamma$ .....	0.5	0.2	10	200	0.4	1	0.4	0.4	S	1	0.2	1	0.2	0.2
Adenine sulfate, mg.....	1.0	1.0	2.0	1.2	2.4	1.0	1.2	1.0	5	1	1.0	1	1.0	1.2
Guanine-HCl, mg.....	1.0	1.0		1.2	2.4	1.0	1.2	1.0	5	1	1.0	1	1.0	1.2
Uracil, mg.....	1.0	1.0		1.2	2.4	1.0	1.2	1.0	5	1	1.0	1	1.0	1.2
Xanthine, mg.....	1.0					1.0								
Alanine, mg.....	20*	20*	20*	200*	400*	20*	100*	20*	40*	20*	20*	20*	20*	100*
Aspartic acid, mg.....				†	†	40	200*	40*	40	40	20*	40	20*	
Asparagine, mg.....	40	20*	20*	40	80	10	20	10	20	10	20	10	20	40
Arginine-HCl, mg.....	10	20	20	8	16	10	20	10	20	10	20	10	20	40
Cystine, mg.....	10	20	20	12	24	10	20	10	20	10	20	10	20	12
Glutamic Acid, mg.....	50	20*	20*	15	30	100	80*	40*	40	100	20*	100	20*	15
Glycine, mg.....	10	20	20	10	20	10	10	10	20	10	20	10	20	10
Histidine-HCl·H <sub>2</sub> O, mg.....	10	20	20	10	4	10	10	10	20	10	20	10	20	2
Hydroxyproline, mg.....														
Isoleucine, mg.....	20*	20*	20*	15*	30*	20*	20*	20*	20*	10*	20*	10*	20*	15*
Leucine, mg.....	10	20*	20*	7.5	15	10	20*	20*	20	10	20*	10	20*	7.5
Lysine-HCl, mg.....	20	10	20*	18*	32*	10	20	10	20*	10	20*	10	10	16
Methionine, mg.....	20*	20*	20*	4*	8*	20*	10*	20*	20*	20*	20*	20*	20*	4*
Norleucine, mg.....				10*	20*	20*	20*	20*	20*	20*	20*	20*	20*	
Norvaline, mg.....				10*	20*	20*	20*	20*	20*	20*	20*	20*	20*	6*
Phenylalanine, mg.....	20*	20*	20*	6*	12*	20*	10*	20*	20*	20*	20*	20*	20*	6*
Proline, mg.....	10	20	20	2.5	5	20*	10	10	20	10	20	10	20	2.5
Serine, mg.....	10	20*	20*	8*	16*	20*	†	†	20*	20*	†	20*	†	8*
Threonine, mg.....	20*	20*	20*	45*	90*	20*	†	†	20*	20*	†	20*	†	45*
Tryptophan, mg.....	10*	40*	20	1.0	2	10	5	10	20	10	40*	10	40*	1.0
Tyrosine, mg.....	10	20	20	3.0	6	10	10	10	20	10	20	10	20	3.0
Valine, mg.....	20*	20*	20*	15*	30*	20*	20*	20*	20*	20*	20*	20*	20*	15*
Casein (acid hydrolyzate), gm.....														
Organism.....	F	F	F	M	M	A	M	M	M	M	M	C	D	M

TABLE 9—Concluded

\* DL—form.

Italics—denotes amino acid required by organism.

Bold face or †—denotes amino acid determined.

A = *L. arabinosus* 17-5; B = Lactic acid bacteria, 23 strains; B' = Lactic acid bacteria, 8 strains. In some experiments sodium citrate substituted for sodium acetate P-60; M' = *L. mesenteroides*, No. 535; P = *L. pentosus*; S = Supplement; T = *E. typhosa*.  
 a = Glutamine, 2.5 mg.; b = Glutamine, required; c = Plus xylose, 0.1 gm.; d = Pyridoxamine-2HCl, 40 gamma; e = Plus succinic acid, 1 gm.; f = Plus sodium citrate, 2 gm.; g = Glutamine determined; h = Plus pyridoxamine-2HCl, 120 gamma; i = Pyridoxamine-2HCl, 40 gamma; k = Plus pyridoxal, 2 gamma; l = Plus pyridoxamine-2HCl, 10 gamma and pyridoxal, 10 gamma; m = Plus glutamine, 0.5 mg.; p = Cysteine-HCl, a = Free base; r = H<sub>2</sub>O<sub>2</sub> oxidized; s = Peptone, H<sub>2</sub>O<sub>2</sub> oxidized; t = Plus succinic acid, 1 gm.; u = Pyridoxal; v = Plus casein tryptic digest, 0.06 gm.; z = Sodium citrate; y = Sodium acetate-3H<sub>2</sub>O; s = Pyridoxamine-2HCl, 20 gamma.

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## REFERENCES—TABLE 9—Continued

- fibrin, edestin, horse hemoglobin, and horse carboxyhemoglobin. Glutamine also determined with *L. arabinosus* 17-5. Aspartic acid determined with *L. mesenteroides* P-60 in the same proteins. Glutamine more active than glutamic acid for *L. arabinosus* 17-5, while aspartic acid more active than asparagine for *L. mesenteroides* P-60.
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- valine, phenylalanine, arginine and tyrosine. Using a variety of media it was found that the D-isomers of leucine and valine were inactive for all three organisms. Methionine and tryptophan were inactive for *S. faecalis* R. and *L. arabinosus* 17-5. Lysine was inactive for *S. faecalis* R. Folic acid prepared from liver powder concentrate extract.
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these amino acids was required for growth of the organism on an enriched basal medium of the composition shown in table 7. Confirmatory evidence may be needed, therefore, before the reliability of the values for these amino acids in natural products can be accepted without reservation.

In 1945 and 1946 Stokes *et al.* (51, 166, 167) described procedures for the determination of 9 amino acids with a single basal medium (No. 32, table 9) using one organism (*S. fecalis* R). As shown in table 12, later workers have followed this trend although there has been a tendency to employ *L. mesenteroides* P-60 in conducting assays in this manner. From 5 to 15 amino acids were determined with this organism in each of five laboratories referred to in this table. The question still remains, however, whether or not all, or any large number of amino acids, can be determined as satisfactorily in this way as by the use of different basal media and organisms. The data shown in table 13 indicate the variations in the composition of the basal media (table 9) which have been employed for the determination of amino acids with *L. mesenteroides* P-60. It has not been determined, however,

TABLE 10. AMINO ACIDS WHICH HAVE BEEN DETERMINED WITH LACTIC ACID BACTERIA<sup>1</sup>

ORGANISM	ALANINE	ARGININE	ASPARTIC ACID	CYSTINE	GLUTAMIC ACID	GLYCINE	HISTIDINE	HYDROXY PROLINE	ISOLEUCINE	LEUCINE	LYSINE	METHIONINE	PHENYLALANINE	PROLINE	SERINE	THREONINE	TRYPTOPHAN	TYROSINE	VALINE
<i>L. arabinosus</i> 17-5.....				X	X				X	X		X	X			X	X	X	X
<i>L. casei</i> <sup>2</sup> .....		X	X		X				X	X			X		X		X	X	X
<i>S. faecalis</i> R.....	X	X	X		X		X		X	X	X	X			X	X	X	X	X
<i>L. fermenti</i> 36.....							X					X				X			
<i>L. mesenteroides</i> P-60..	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X

X Unpublished experiments, Rockland, L. B. and M. S. Dunn.

<sup>1</sup> Data taken from table 9 and Dunn, M. S. and L. B. Rockland. *Advances in Protein Chemistry* 3: 295, 1947.

<sup>2</sup> Same as *L. delbrueckii* according to Rogosa (139) and Dunn (34).

whether or not assays with these different media would yield results of the same or markedly different precision and accuracy.

The experiment whose results are summarized in table 14 was undertaken in the writer's laboratory in order to compare different organisms and basal media in assays of amino acid test mixtures and casein. Although the recoveries of threonine from 4 amino acid test mixtures containing from 0.5 to 4.0 per cent of threonine ranged from about 94 to 104 per cent, the values found for the percentage of threonine in casein varied only from 4.24 to 4.36 per cent. It seems probable that the agreement would be somewhat better if this experiment were to be performed according to the improved techniques now available.

The data from another experiment of interest in this connection are summarized in table 15. A test mixture containing alanine and serine in addition to the 16 amino acids listed in the table was assayed in each of six laboratories by the microbiological procedure in vogue. It may be noted that alanine and serine were not determined, that the values reported for cystine, glycine and proline are not satisfactory and that

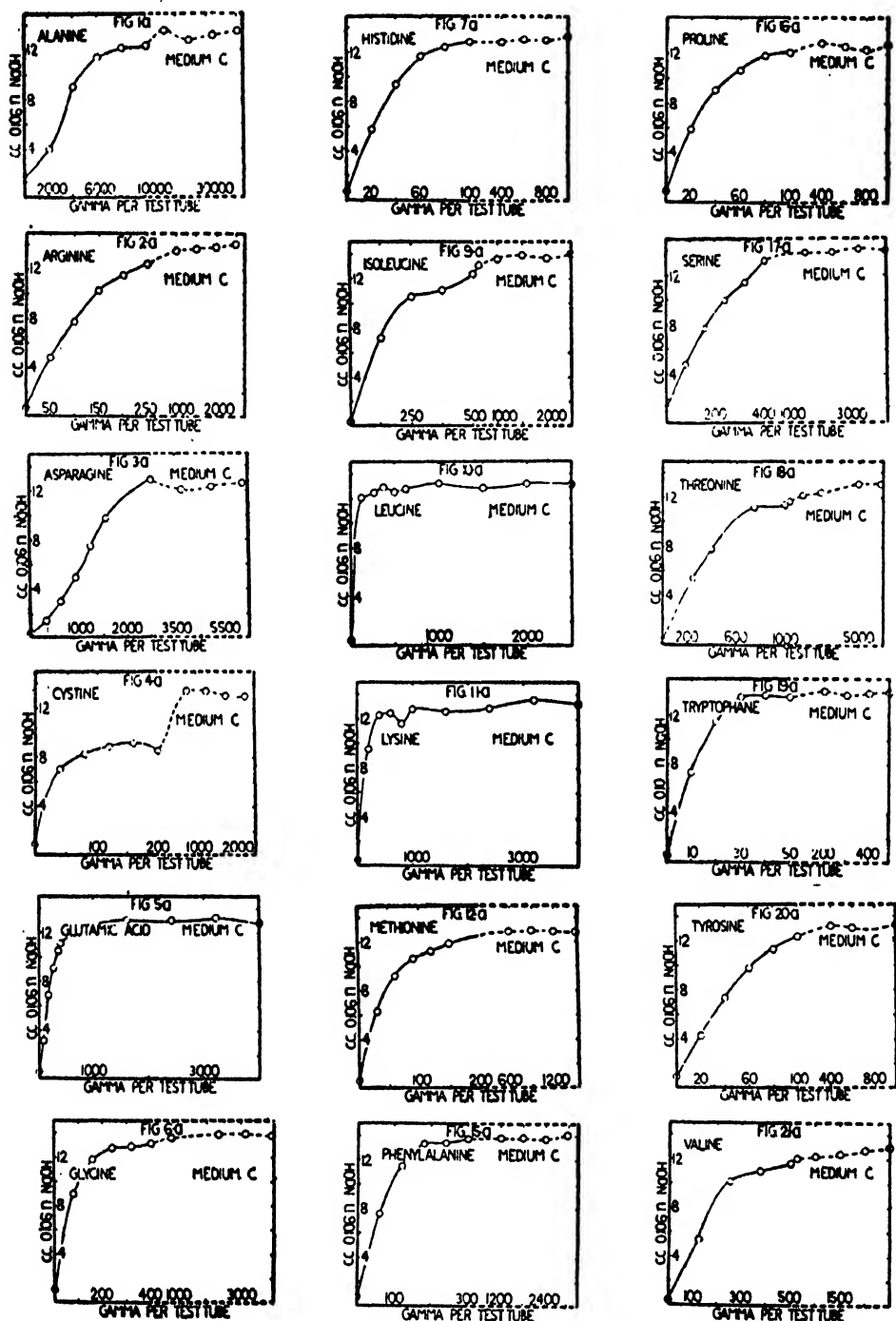


Fig. 3. STANDARD CURVES for 18 amino acids essential for growth of *L. mesenteroides* P-60 (DUNN, M. S., S. SHANKMAN, M. N. CAMIEN, W. FRANKL AND L. B. ROCKLAND. *J. Biol. Chem.* 156: 703, 1944).

acceptable values were obtained only in about half of the laboratories for arginine, histidine, lysine, threonine and tryptophan. There is a possibility, however, that some of the recoveries may prove to be better than those recorded in the table since there has been no opportunity to make certain that all of the values have been calcu-

TABLE II. AMINO ACIDS DETERMINED IN NATURAL PRODUCTS WITH *L. mesenteroides* P-60

AMINO ACID	NATURAL PRODUCT	REF. NO.
Alanine.....	Corn steep liquor	1
Arginine.....	Corn steep liquor, enzymes	1, 2
Aspartic acid.....	Food proteins, viruses, bacterial toxins, enzymes	1-5, 16, 17
Cystine.....	Corn steep liquor, urine, enzymes	1, 2, 5
Glutamic acid.....	Enzymes	2
Glycine.....	Casein, silk fibroin, viruses, bacterial toxins, enzymes	2, 6, 16, 17
Histidine.....	Food proteins, viruses, bacterial toxins, enzymes	1, 2, 4, 7, 8, 9, 10 11, 17
Isoleucine.....	Food proteins, enzymes	2, 4
Leucine.....	Enzymes	2
Lysine.....	Corn steep liquor, food proteins, urine, enzymes, viruses, bacterial toxins	1, 3, 4, 5, 7-9, 12, 13, 16, 17
Methionine.....	Corn steep liquor, wheat, enzymes	1-3, 8
Phenylalanine.....	Corn steep liquor, enzymes	1-3, 8, 14
Proline.....	Corn steep liquor, enzymes, viruses, bacterial toxins	1-5, 15-17
Serine.....	Corn steep liquor, enzymes, urine	1, 2, 5
Threonine.....	No assays reported	—
Tryptophan.....	Food proteins	Author, unpubl.
Tyrosine.....	Food proteins, enzymes, urine	2, 4, 5
Valine.....	Enzymes	2

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lated on the same basis. The most striking conclusions to be drawn from this experiment are that the recoveries averaged 101 per cent for 66 of the 79 amino acid determinations and that about 80 per cent of the 66 values were within  $100 \pm 3$  per cent of the correct figures. These findings strongly support the belief that many, if not all, amino acids can be determined with sufficient accuracy for many purposes

using different organisms and different basal media. It is recognized, however, that there is urgent need for improvement in basal media and methods applicable to the determination of certain amino acids in the assay of purified proteins, physiological fluids and other materials of theoretical and practical importance.

The composition of basal media employed currently for amino acid assays has been determined more or less arbitrarily. The concentration of carbohydrate,

TABLE 12. LACTIC ACID BACTERIA USED TO DETERMINE AMINO ACIDS IN SIX LABORATORIES

AMINO ACID	BARTON- WRIGHT	CARD- INALL & HEDRICK	DUNN <i>et al.</i>	HENDERSON & SNELL	STOKES <i>et al.</i>	VELICK & RONZONI
Alanine.....		M				
Arginine.....	F	M	C	F or D	F	M
Aspartic acid.....	M	M	M	M	D	M
Cystine.....	A	M	A or M			M or A
Glutamic acid.....		A	A	A		M
Glycine.....			M			M
Histidine.....	M	M	M	F or M	F	M
Isoleucine.....	A	A	A	M	F	M or A
Leucine.....	A	A	A	A	F	M or A
Lysine.....	M	M	M	M	F	M
Methionine.....	M	M	A or FER.	F	F	M or FER.
Phenylalanine.....	M	M	M	A	D	M
Proline.....	M	M	P*	M		M
Serine.....		M			D	M
Threonine.....	F	F	FER.	F	F	FER.
Tryptophan.....	A		A	A	F	
Tyrosine.....			C	M	D	M
Valine.....	A	A	A	A	F	M or A

Notations: A, *L. arabinosus* 17-5; C, *L. casei*; D, *L. delbrueckii*; F, *S. faecalis* R; FER., *L. fermenti* 36; M, *L. mesenteroides* P-50; P, *L. brevis*.

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ammonium chloride, amino acid, phosphate buffer and inorganic salts in Uschinsky's original (1893) medium has been changed somewhat and numerous other nutrients have been added. Types and proportions of phosphate and other inorganic salts, essentially the same as those proposed by Speakman (163) in 1923, have been adopted by most workers. Following the isolation of thiamine (63) in 1926, vitamins were introduced into basal media as soon as they became available in crystalline form.

Basal media containing as many as 19 amino acids have been employed since 1922 to grow microorganisms and since 1936 to cultivate lactic acid bacteria. Although

TABLE 13. RANGE OF CONCENTRATIONS OF NUTRIENTS IN BASAL MEDIA USED TO DETERMINE AMINO ACIDS WITH *LEUCONOSTOC MESAENTEROIDES* P-60

	%		%		%
Glucose.....	1-4	Biotin.....	0.1-1.0	DL-Isoleucine.....	15-40
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .....	0-0.6	Folic acid.....	0.1-1.0	L-Leucine.....	7.5-20
NaAc.....	0.6-2.4	Pyridoxamine·HCl....	0-120	DL-Leucine.....	20-20
NH <sub>4</sub> Cl.....	0-1.2	Adenine sulfate·2H <sub>2</sub> O..	1.0-2.4	L-Lysine·HCl.....	10-32
KH <sub>2</sub> PO <sub>4</sub> .....	0.05-1.6	Guanine·HCl·2H <sub>2</sub> O....	1.0-2.4	DL-Lysine·HCl....	10-32
K <sub>2</sub> HPO <sub>4</sub> .....	0.05-1.6	Uracil.....	1.0-2.4	DL-Methionine.....	4-20
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.02-0.04	Xanthine.....	0-2.0	DL-Norleucine.....	0-40
NaCl.....	0.001-0.5	DL-Alanine.....	20-400	DL-Norvaline.....	0-40
FeSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.001-0.002	L-Aspartic acid.....	40-80	DL-Phenylalanine...	6-40
MnSO <sub>4</sub> ·4H <sub>2</sub> O.....	0.001-0.002	DL-Aspartic acid.....	40-200	L-Proline.....	2.5-20
Inositol.....	0-250	L-Asparagine.....	20-80	DL-Proline.....	20-20
Choline chloride...	0-250	L-Arginine·HCl.....	8-60	DL-Serine.....	8-40
Thiamine chloride..	50-200	L-Cysteine·HCl.....	16-16	DL-Threonine.....	20-90
Pyridoxine·HCl....	100-1000	L-Cystine.....	10-24	L-Tryptophan.....	1-20
Ca pantothenate...	20-400	L-Glutamic acid.....	15-200	DL-Tryptophan....	10-10
P-Aminobenzoic acid.....	0.01-50	Glycine.....	10-20	L-Tyrosine.....	3-10
Riboflavin.....	20-400	L-Histidine·HCl·H <sub>2</sub> O..	2-20	DL-Tyrosine.....	10-10
Nicotinic acid.....	20-400	L-Hydroxyproline.....	0-20	DL-Valine.....	15-40

TABLE 14. THREONINE IN FOUR AMINO ACID TEST MIXTURES<sup>1</sup> AND CASEIN<sup>2</sup> DETERMINED WITH THREE LACTIC ACID BACTERIA AND FOUR BASAL MEDIA

SAMPLE	S. FAECALIS R		L. ARAB- INOSUS	L. FERMENTI
	Stokes	Greenhut	Hier	Dunn
Test mixt., 0.5% threonine.....	93.5	104	97.5	97.7
Test mixt., 1.0% threonine.....	94.5	101	97.5	97.9
Test mixt., 2.0% threonine.....	93.4	104	98.5	96.0
Test mixt., 4.0% threonine.....	93.1	104	100	94.8
Casein, moisture-ash free.....	4.25	4.36	4.36	4.24

<sup>1</sup> Values given as per cent recovery.      <sup>2</sup> Per cent threonine.

<sup>3</sup> Data in table obtained in writer's laboratory.

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reasonably satisfactory assay procedures for the determination of amino acids have been elaborated, it is not to be assumed that any available method approaches perfection.



A study of basal media to be used in the quantitative determination of amino acids in protein materials was initiated by Dunn and co-workers (35) in 1944. Although it was desired that this investigation should be systematic and complete, rather than empirical and limited, it was recognized that a prohibitive length of time would be required to determine the environmental conditions and the types, proportions and levels of nutrients which are optimal for the growth of lactic acid bacteria. In this work the minimum quantity of each amino acid required to give

TABLE 15. DATA FOR RECOVERY OF AMINO ACIDS FROM TEST MIXTURE DETERMINED BY MICROBIOLOGICAL ASSAY IN SIX LABORATORIES

AMINO ACID	% RECOVERY BY LABORATORY						AMINO ACID	% RECOVERY BY LABORATORY					
	1	2	3	4	5	6		1	2	3	4	5	6
Arginine.....	98	113	94	107	84	103	Lysine.....	101	103	104	87	78	104
Aspartic acid.....	99	99		98		102	Methionine.....	99	109	101	100	99	102
Cystine.....		76				127	Phenylalanine.....	103	100	99	103	101	100
Glutamic acid.....	101	101		100	105	100	Proline.....		117		101		
Glycine.....	104					107	Threonine.....	100	100	101	111	114	94
Histidine.....	99	104	102	114	80	108	Tryptophan.....		109	126	100	125	102
Isoleucine.....	99	102	104	99	99	105	Tyrosine.....		99	100	101	109	100
Leucine.....	100	97	99	98	90	101	Valine.....	101	103	98	101	99	103
Range <sup>1</sup> .....								98-104	97-109	94-104	98-107	99-109	94-108
Average <sup>1</sup> .....								100	102	100	101	102	102

<sup>1</sup> Italic values omitted in determining ranges and calculating averages.

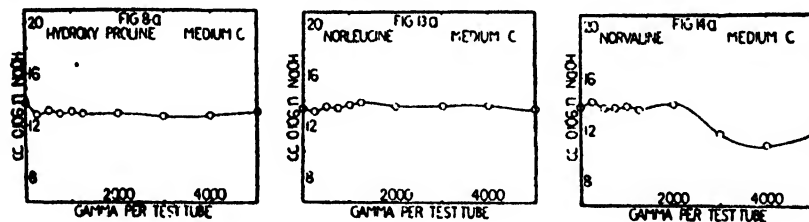


Fig. 4. STANDARD CURVES for non-essential amino acids, hydroxyproline, norleucine and norvaline (DUNN, M. S., S. SHANKMAN, M. N. CAMIEN, W. FRANKL AND L. B. ROCKLAND. *J. Biol. Chem.* 156: 703, 1944).

the maximum titration and the minimum level of the amino acids as a whole at which the slope of the standard curve for a particular amino acid was maximum, were determined using a basal medium of arbitrary composition. Then a new basal medium was prepared of the same composition as the original except that each essential amino acid was introduced at the minimum level found to be optimum. If the minimum amounts of an amino acid required to give maximum titrations did not differ significantly in the two experiments, it was assumed that the indicated proportion of the amino acid was optimum. If necessary, this experimental procedure was repeated

until a constant minimum level of each amino acid resulted. Finally, the composition of the near-optimum basal medium was determined from these data.

The amino acid requirements of *Leuconostoc mesenteroides* P-60 (35), *Lactobacillus fermenti* 36 (26) and about 20 other lactic acid bacteria (34), as well as some of the vitamin (148) and carbohydrate (17) requirements of these organisms, have been determined in this manner. It has been possible to develop assay procedures for the determination of glutamic acid (25), glycine (149), histidine (30, 31, 33), leucine (16), lysine (29), methionine (27), phenylalanine (32), threonine (28) and some other amino acids for which methods have not been reported. A microbiological procedure for the determination of proline with *Lactobacillus brevis* is to be described in a forthcoming paper.<sup>8</sup> It is of interest in this connection that Boyd *et al.* (8, 9) have recently determined the amino acid requirements of *Clostridium per-*

TABLE 16. ORGANISMS OTHER THAN LACTIC ACID BACTERIA USED TO DETERMINE AMINO ACIDS

AMINO ACID	ORGANISM	REF. NO.	AMINO ACID	ORGANISM	REF. NO.
Amino acids (thirteen) . . .	<i>Cl. perfringens</i> BP6K	(1)	Methionine. . .	<i>E. coli</i> 532-171	(3, 9)
Arginine. . . . .	<i>E. coli</i> 1577-28	(2, 3)	Phenylalanine. .	<i>E. coli</i> mutants	(10)
Histidine. . . . .	<i>Tetrahymena geleii</i> H	(4)	Tryptophan. . .	<i>E. coli</i> mutant	(3)
Leucine. . . . .	<i>Neurospora crassa</i> 33757	(5-7)	Tryptophan. . .	<i>Tetrahymena geleii</i> H	(11)
Leucine. . . . .	<i>E. coli</i> 679-680	(8)	Tyrosine. . . . .	<i>E. coli</i> 58-5030	(10)

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*fringens* (Welchii) BP6K and have reported a procedure for the determination of 13 amino acids with this organism. A possible disadvantage may be that this strain of *Clostridium* is potentially a toxin former. A microbiological assay procedure for alonine has been reported by Sauberlich and Baumann.<sup>9</sup>

Since it has been established that microbiological data for amino acids vary in precision and accuracy depending upon the amino acid determined, the protein or other amino acid mixture assayed, the lactobacillus strain used as the test organism, and the control exercised over the environmental conditions it may be that basal media whose composition has been determined on logical premises may yield analytical results of higher quality than those obtained under other conditions. On the other hand it may very well be true that acceptable data for some, if not all, purposes

<sup>8</sup> See table 12 for reference to proline paper.

<sup>9</sup> Sauberlich, H. E. and C. E. Baumann. *J. Biol. Chem.* 177: 545, 1949.

TABLE 17. TOTAL AND 'FREE' AMINO ACIDS IN NORMAL URINE

AMINO ACID	TOTAL		FREE		AMINO ACID	TOTAL		FREE	
	(1)	(2)	(3)	(4)		(1)	(2)	(3)	(4)
Arginine.....	30	24	9.5	21	Leucine.....	29	21	7.2	9.6
Aspartic acid.....	175	165		1.3	Lysine.....	84	73	47	34
Cystine.....			108(4)	88	Methionine.....	14	8.6	4.4	7.8
Glutamic acid.....	347	351		36	Phenylalanine.....	29	23	14	16
Glycine.....	714				Threonine.....	49	54	21	24
Histidine.....	170	203	163	188	Tryptophan*.....			18(4), 20(5)	25
Isoleucine.....	10	20	14	5.0	Valine.....	27	20	7.4	4.5

\* Albanese and Frankston (6) found 281 mg. and Berg and Rohse (7) found 164 and 26 mg. of tryptophan in normal urine by colorimetric methods.

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TABLE 18. PERCENTAGE OF AMINO ACIDS IN ORGANS OF MAMMALS AND BIRDS<sup>1</sup>

AMINO ACID	BLOOD	CARCASS	HEART	KIDNEY	LIVER	MUSCLE	SKIN
Arginine							
mammal.....	3.6-4.4	4.8-5.6	5.2-6.4	5.2-6.3	5.4-6.5	5.4-6.3	7.6-8.8
bird.....	4.7-5.1	5.3-5.9	5.3-6.4	5.6-6.7	5.1-6.7	5.7-6.1	5.3-7.0
Aspartic acid							
mammal.....	11-14	7.8-10	10-11	9.7-11	10-12	9.7-11	6.5-7.5
bird.....	9.8-11	7.6-10	10-11	9.5-11	10-11	9.7-11	6.3-9.3
Glutamic acid							
mammal.....	7.6-12	11-14	9.3-15	11-12	14-15	16-18	11-14
bird.....	9.5-10	11-12	13-16	11-12	14-16	16-18	10-11
Glycine							
mammal.....	3.5-6.9	6.9-10	5.5-9.4	5.4-7.3	5.2-6.5	4.3-7.1	12-15
bird.....	2.8-4.4	7.7-13	5.0-5.8	4.5-6.0	5.2-7.8	4.6-6.7	8.0-13
Histidine							
mammal.....	5.7-7.5	1.7-2.0	2.5-3.6	2.3-4.2	2.5-2.9	1.9-2.4	1.1-1.4
bird.....	5.4-5.9	1.7-2.0	2.5-2.9	2.7-3.6	2.7-3.0	2.2-2.3	0.6-1.2
Isoleucine							
mammal.....	1.7-3.6	3.4-4.4	4.2-5.2	4.0-4.8	5.0-5.7	4.3-5.3	2.2-2.6
bird.....	4.4-4.9	3.7-4.5	4.9-5.4	4.6-5.4	5.2-6.2	4.6-5.2	3.4-6.2

<sup>1</sup> Mammals: Cat, dog, mouse, rabbit and rat. Birds: Chicken, duck and turkey. Values corrected to 16% nitrogen. Data from Dunn *et al.*, *U. Calif. Publ. Physiol.* 8: 293, 1949.

can be acquired using basal media varying widely in the types and proportions of their nutrient substances.

The following questions are examples of those which must be answered before one can determine the quality of the amino acid data obtained for proteins, foods and physiological fluids. What stimulatory and inhibitory influences on the growth of lactic acid bacteria are exerted by various derivatives, analogs, homologs and antipodes of the amino acids in blood, urine and protein hydrolyzates? What is the effect of peptides (including streptogenin) on the growth of these organisms? How can cystine, tryptophan and some other labile amino acids be liberated from proteins without undergoing the destruction resulting from acid or base hydrolysis? What reactions occur between carbohydrates, vitamins, amino acids and other nutrients during autoclaving of basal media and to what extent do they lower the precision

TABLE 19. PERCENTAGE OF AMINO ACIDS IN ORGANS OF MAMMALS AND BIRDS<sup>1</sup>

AMINO ACID	BLOOD	CARCASS	HEART	KIDNEY	LIVER	MUSCLE	SKIN
<b>Leucine</b>							
mammal.....	11-13	6.2-7.1	7.8-13	7.6-8.8	8.9-9.9	7.0-8.1	4.2-5.6
bird.....	10-11	6.7-7.8	8.2-9.0	8.3-9.4	9.2-10	7.3-7.8	6.6-7.9
<b>Lysine</b>							
mammal.....	9.2-11	5.5-9.0	7.6-8.9	6.7-7.7	6.9-8.2	7.7-9.0	3.7-5.2
bird.....	8.3-10	6.4-8.9	7.0-8.2	7.6-8.3	7.3-8.0	8.3-8.8	2.4-5.0
<b>Methionine</b>							
mammal.....	0.8-1.8	2.0-2.5	1.6-2.5	2.0-2.5	2.0-2.4	2.3-2.7	0.9-1.2
bird.....	1.2-1.6	1.8-2.5	2.2-2.9	2.0-2.2	2.0-2.4	2.3-2.7	0.8-1.1
<b>Phenylalanine</b>							
mammal.....	6.6-8.0	3.1-3.7	4.2-4.9	3.5-4.8	4.8-5.5	3.5-4.0	2.3-3.0
bird.....	5.8-6.7	3.0-3.6	4.1-4.8	4.7-5.3	4.8-5.1	3.7-3.9	3.8-4.6
<b>Threonine</b>							
mammal.....	4.5-6.3	3.6-4.0	4.4-4.8	4.0-4.8	4.3-5.0	3.9-5.0	3.0-4.2
bird.....	4.6-5.3	2.7-4.0	3.9-4.9	3.9-5.0	3.6-4.7	3.5-4.5	2.8-5.1
<b>Valine</b>							
mammal.....	7.2-8.8	4.2-5.0	4.7-5.9	5.4-6.4	6.1-6.6	4.5-5.1	3.3-4.2
bird.....	7.0-8.0	4.1-4.8	5.6-6.2	5.9-6.5	6.4-6.7	4.7-4.9	5.8-7.9

<sup>1</sup> *Mammals*: Cat, dog, mouse, rabbit and rat. *Birds*: Chicken, duck and turkey. Values corrected to 16% nitrogen. Data from Dunn *et al.*, *U. Calif. Publ. Physiol.* 8: 293, 1949.

and accuracy of amino acid assay data? How can any difficulties of this kind be circumvented? Can amino acid assays be made with as much, or greater, convenience and dependability with *Tetrahymena*, *Clostridium*, mutants of *Neurospora crassa* or *E. coli* and other organisms of the types shown in table 16?

Despite any shortcomings inherent in the microbiological procedures available today for the determination of amino acids, it is evident that these methods are being used increasingly and that they have almost unlimited application to the determination of amino acids in many, if not all, types of biological materials. Examples are given in table 17 of the amino acid data for normal urine obtained in 3 laboratories and values for amino acids in different organs and tissues of a number

of laboratory or domestic mammals and birds are shown in tables 18 and 19. It would not have been feasible, previously, to undertake studies of such scope.

It may be said, finally, that the developments in the microbiological assay of amino acids which have been discussed may serve to illustrate anew the statement that "*The greatest advances come, not from the refinement of old methods, but by the discovery of new tools.*"

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# EXOPHTHALMOS

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**F**OLLOWING THE DESCRIPTIONS of exophthalmic goiter by Plummer (97), Graves (41), Basedow (9) and others, many workers have studied the production of exophthalmos in lower animals. Their results have made clear the paths from cerebral neurones to the smooth muscle and vessels of the orbit. They have laid sound foundations for the treatment of exophthalmos in lower animals. Though their results cannot be applied directly to cases of exophthalmos in man owing to anatomical differences in the human orbit, the production of exophthalmos by an anterior pituitary principle is of great interest to physicians and surgeons.

## EXPERIMENTAL EXOPHTHALMOS IN LOWER ANIMALS

Perhaps the first worker to throw light on the mechanism of exophthalmos was Claude Bernard (11). He showed that if the cervical sympathetic nerve trunk in the dog was cut, the eyeball receded and the upper lid drooped. If the peripheral (cephalic) end of the cut nerve was stimulated electrically, these effects were reversed. In later work Bernard (12) showed the spinal segments from which fibers pass in the cervical sympathetic trunk to the pupil and to the vessels of the head, but he made no report on the fibers which altered the position of the globe in relation to the orbit.

In 1858 Müller (85) published a 'preliminary communication' on the smooth muscle of the orbit in men and animals. The first two paragraphs of it may be translated as follows:

In man the inferior orbital fissure is filled with a greyish red mass. This consists of bundles of smooth muscle fibers which are usually equipped with elastic tendons. In mammals there is a strongly developed muscular membrane connected with elastic laminae which is its more strongly developed analogue and which consists also of smooth muscle fibers (musculus orbitalis or orbital membrane of the author).

After a paragraph on the nictitating membrane the communication concludes:

The orbital muscle is supplied with nerve bundles which consist almost entirely of fine or non-medullated (sympathetic) fibers. The nerves can be traced anatomically in part to the sphenopalatine ganglion. The orbital muscle produces, by its contraction, the protrusion of the globe which has been observed in animals during the stimulation of the cervical sympathetic nerve. The same muscle acts as antagonist to the muscles which move the globe backwards in its cavity (M. retractor, orbicularis palpebrarum).

Müller's untimely death shortly afterwards prevented him from writing a fuller account of this orbital muscle or membrane which has been known since as 'Müller's orbital muscle' and is to be distinguished from the smooth-fibered part of the levator palpebrae superioris or superior tarsal muscle (100) to which the name of Müller's palpebral muscle is often given. Excepting the figure from Harling's article (44),

no illustrations of this muscle were available when the present writer was studying differences between the muscles of lower mammalian and human orbits. Photomicrographs of the muscle in the dog and cat are given by Brunton (18).

Harling (44) studied the orbital muscle in the sheep, dog, and man and illustrated the muscle in sheep. He could not believe that the muscle in the infra-orbital fissure of man is able to draw the eye forward and most later writers (134) agree with this conclusion. In the dog and other lower mammals, however, the orbital muscle is a hollow cone with its apex near the optic foramen and sphenoidal canal and its base attached to the anterior orbital ring. Prévost and Jolyet (103) showed that the orbital muscle contracted when the cervical sympathetic nerve trunk was stimulated.

MacCallum and Cornell (69) published a classical account of their investigations on exophthalmos which described both dissections of human and canine orbits and a number of recorded responses to stimulation of the cervical sympathetic nerve trunk. In experiments on the dog they removed the roof, external wall and much fatty tissue from the orbit. This enabled them to observe the contraction of Müller's orbital muscle. They recorded the movements of the eyeball on a moving drum by a lever. One end of this lever was fitted with a writing point and the other end lay on the dog's cornea. In this way they avoided the illusion of exophthalmos which can be produced by lid retraction. The contraction of the muscular cone could be seen during stimulation of the nerve and the movement of the globe was recorded on the drum by the writing point of the lever. When they replaced the eyeball by a balloon, stimulation of the cervical sympathetic nerve trunk increased the pressure in the balloon so that it advanced in the orbit, and thus showed that no muscular attachment was necessary to draw the eyeball forward. When the position of the balloon was kept constant, its internal pressure increased when the nerve was stimulated. In other experiments MacCallum and Cornell produced exophthalmos by obstructing the venous outflow from the orbit. They also showed in the cat that stimulation of the ventral root of the second thoracic nerve produced both exophthalmos and pupillary dilatation. Whitnall and Beattie (135) obtained results on cats similar to those of MacCallum and Cornell on dogs. Using spinal dogs under chloralose Code and Essex (24) demonstrated by motion photography the response of Müller's orbital muscle and of the eyeball to stimulation of the cervical sympathetic trunk. Stimulation of the vago-sympathetic trunk has failed to produce exophthalmos in apes and in human subjects (10, 60, 61, 69, 99). Indeed the mechanism of exophthalmos in man often remains doubtful. In the horse, sheep, dog, cat, rabbit and guinea-pig, however, the immediate cause of exophthalmos may be due either to Müller's orbital muscle or to changes in the blood supply of the orbit (19). Events in the body which influence these mechanisms will be considered next.

The path of nerve fibers beyond the superior cervical ganglion has been little studied since Müller stated that some fibers supplying his orbital muscle could be traced to the sphenopalatine ganglion. (His article did not show whether this had been done in man or a lower mammal.) The writer sought for a synapse in the sphenopalatine ganglion by infusing eserinated Ringer's solution containing acetylcholine into the artery which supplied the ganglion in the isolated head of the dog. After a few experiments the work was interrupted by the Second World War but a

provisional conclusion was reached that no post-ganglionic fibers originated at this site. The work did not disprove a hypothesis that fibers might pass through the ganglion without being relayed. Recently an article by de Kleijn and Socin (28) has come to the notice of the writer. These workers studied the post-ganglionic nerves to the nictitating membrane, the pupil and the orbital vessels in the cat. By a series of nerve sections and stimulations they traced these nerve fibers away from the internal carotid artery laterally into the middle ear, forward alongside but not in the wall of the eustacian tube to the foramen rotundum, piercing the basiphenoid lateral to the vidian nerve and medial to the second root of the trigeminal nerve and then either with the first part of the trigeminal nerve to the pupil or with some other nerve to the nictitating membrane and the smooth retractor muscle of the upper lids. Further study of this difficult path would be rewarding. It seems possible that the fibers to Müller's orbital muscle travel by the same path as these for the retractor of the upper lid.

The possibility of a nervous pathway from the stellate ganglion along the vertebral artery to the orbit was suggested by observations of Leriche and Fontaine (67) on human subjects. They noticed dilatation of the pupil after the cervical sympathetic trunk had been cut. This effect, however, might have been produced either by a rise of blood pressure in the orbital cavity or by a rise in the adrenalin content of the blood. Each of these conditions may be produced under normal conditions by emotion. A recession of the globe when dogs under anesthesia are spinalized has been noticed by Code (23) but this recession may depend upon a fall of general body blood pressure. Brunton (19) recorded the effect produced on the eyeball by stimulation of the stellate ganglion before and after cutting the cervical sympathetic trunk in dogs. No response in the orbit was obtained after cutting the nerve trunk, though stimulation of the cut distal end of the trunk still produced exophthalmos. The conclusion was drawn that usually no path for production of exophthalmos originated in or passed through the stellate ganglion in the dog except that carried by the fibers of the cervical sympathetic trunk.

The nerve path to Müller's orbital muscle has been gradually traced, in certain animals at least, from the cervical nerve trunk to the anterior root of the second thoracic nerve (69) to the region of the fourth ventricle (36), to the hypothalamic region and almost certainly in the cat to the frontal lobe of the brain. Following the work of Karplus and Kreidl (56, 57, 58), Bard (6, 7) studied the effects caused in cats by loss of the cerebral hemispheres, the corpora striata and the anterior half of the hypothalamic region with the corresponding part of the thalamus. Among the motor activities which followed the operation was exophthalmos. Bard suggested that the exophthalmos was due to release of posterior hypothalamic and lower centers from control by the brain areas which had been removed. Further work (42, 49) has shown the existence of sympathetic and parasympathetic centers in the hypothalamic region of the cat and monkey for control of the heart rate, of arterial caliber, of respiration, of heat production and heat loss. The parasympathetic points are anterior to the true sympathetic points. Kennard (55) has traced autonomic paths still farther forward into the frontal lobe of the brain of the monkey where stimulation produces not only somatic motor responses but also such autonomic responses as salivation, lacrimation, pilomotor and vasomotor changes. The study of human

response to emotions and the general integration of the human nervous system has led some to expect this closest possible relation between the two efferent systems at the highest levels. It is probable that a point in the frontal lobe will be discovered which, when stimulated, will produce exophthalmos. Thus the production of exophthalmos by Müller's orbital muscle is almost completely explained.

The production of exophthalmos by changes in the orbital circulation was studied by MacCallum and Cornell (69), by Beattie and Whitnall (135) and by Brunton (19). Interference with the venous outflow or an increase of blood pressure in the isolated head of the dog will produce up to 2.0 mm. of exophthalmos. Acetyl choline will produce exophthalmos by dilation of the orbital vessels but there is no evidence that this occurs in normal animals. In lower mammals the orbicularis and retractor bulbi muscles usually prevent exophthalmos. The retractor muscle has rarely been found in man. One case has been described by Whitnall (133).

So far the influence of endocrine products has not been considered. From the time of Graves (41) and Basedow (9) a connection was suggested between the thyroid gland and the exophthalmos. Sub-total thyroidectomies in man frequently relieved the patients' symptoms, but in a significant proportion of cases exophthalmos persisted after other symptoms had been removed, suggesting that the exophthalmos was not due to excessive thyroid secretion. Nevertheless, other cases were reported in which thyroid medication produced exophthalmos (15) and sub-total thyroidectomies usually removed the patients' troubles. These facts encouraged a study of the results of thyroid administration to lower animals. Early results were complicated by unsatisfactory methods of extraction of thyroid principles and by the unintentional removal of the parathyroid glands from experimental animals. By feeding sheep's thyroid Cunningham (27) produced exophthalmos in rabbits. Edmunds (33, 34) obtained it in rabbits and monkeys; Baruch (8), in bitches; Klose and others (59), in a fox terrier. In 1910 Gley (40) made the important assertion that he had effected exophthalmos in a rabbit after thyroidectomy, a report which was politely ignored until surgeons had reported many cases of exophthalmos after thyroidectomies in human subjects. Using extracts of thyroid glands or Kendall's thyroxin, Kunde (63) obtained slight exophthalmos in normal rabbits, marked exophthalmos in young thyroidectomized rabbits but no exophthalmos in 3 dogs. Although the effect of thyroid substances was still uncertain, the immediate aim of research carried out in the laboratories of the Medical Faculty at Paris was the production and study in dogs of the 'Basedow type of exophthalmos' by means of combinations of sympathomimetic substances including thyroid extract or thyroxin. This type of exophthalmos was defined as "an exophthalmos which involves neither increased tension of the eyeball, orbito-palpebral edema or mydriasis" (52). Dogs reacted to thyroxin in doses of 1 mg/kg/day by loss of weight and tachycardia but showed no exophthalmos. Doses of 0.5 mg/kg of ephedrine were also without effect but a combination of these two in either order produced a rapid and intense exophthalmos (54). The administration of pilocarpine could prevent mydriasis and so produce the Basedow type. Photographs were published and a cinema film was made (53). Boivin (14) found reason for thinking that thyroxin itself, working with adrenalin, actually opposes mydriasis.

Sensitization to adrenalin by denervation has been proved in the case of many

structures which are innervated by sympathetic nerves. Injury to such nerves was at one time a suggested cause of exophthalmos. In 1909 Eppinger and others reported that sensitization of the iris of the dog to adrenalin could be produced by thyroid products. Such sensitization has been reported for the auricle of the guinea-pig heart (62) and for the dog heart (3). The action of adrenalin is extended in time by ephedrine (39); it is potentiated by guanidine (21), by pyrogallol (4) and by acetyl choline (106). Most of this potentiation was proved on the nictitating membrane but the potentiation by pyrogallol has been confirmed by the present writer in respect of exophthalmos in dogs. The fact that exophthalmos produced by adrenalin outlasts the accompanying rise of blood pressure suggested that adrenalin acted apart from the blood pressure but 1) exophthalmos produced by nerve stimulation also outlasts the stimulation (69) and 2) in the isolated head of the dog, if the perfusion pressure is kept constant after injections of adrenalin almost no exophthalmos occurs (19). The exact conditions for the sensitization of exophthalmos by thyroxin are not yet known.

In 1928, Chesney, Clawson and Webster (22) reported that rabbits fed on a diet rich in cabbage developed hyperplastic goiters. In a series of papers, Marine and his co-workers (73-77) reported that exophthalmos occurred in rabbits which were subjected to a cabbage diet; that it occurred more regularly in young male rabbits of Dutch strain and that it could be produced by the injection of 0.1 to 0.25 cc. of methyl cyanide daily for 3 to 8 weeks (77); and that the cyanide produced the exophthalmos after thyroidectomy (74) but failed to do so after removal of the superior cervical ganglion. They suggested in explanation, that the cyanide produced a tissue anoxemia which stimulated the hypothalamic centers. The center then produced impulses which stimulated the anterior pituitary body to produce thyrotropic hormone. This hormone was thought to stimulate a hypothalamic center for exophthalmos besides acting on the thyroid gland (74). It seems possible however, that the anoxemia in the hypothalamic region might stimulate the exophthalmic mechanism without involving the pituitary hormones. The question remains uncertain.

Aron (1) using guinea-pigs first demonstrated the effect of pituitary extract on the mammalian thyroid gland. His work was confirmed by Loeb and Bassett (68) and by many other workers. An admirable review of the work between 1899 and 1938 was published by Lambie (64). Injections of anterior pituitary thyrotropic hormone (APTH) produced exophthalmos as one of their effects and it was found that the hormone produced exophthalmos in rabbits and guinea-pigs even more definitely after thyroidectomy (35, 75, 95, 119). Paulson (95) claims that all his thyroidectomized guinea pigs showed exophthalmos when given APTH. After removal of the superior cervical ganglion, APTH still produced exophthalmos in rabbits (35) and guinea-pigs though cyanide does not act after removal of the ganglion (74).

The changes produced by APTH in the orbit of thyroidectomized guinea-pigs have been studied by Smelser (119). Of 26 pigs (from which the left superior cervical ganglion had also been removed) 23 showed exophthalmos after daily treatment with extracts of 250 mg. of acetone-dried beef anterior pituitary. The exophthalmos was

definite in 12 to 20 days. There was an average increased weight of 100 per cent in the fatty connective tissue of the orbit, of 40 per cent in the dorsal lacrimal gland and of 22 per cent in the extra-ocular muscles. Lymphocytes were present in some cases. Reproductions of photomicrographs were given (120). If Harder's gland were removed from the pig, the increase in weight of the remaining orbital tissues was still 40 per cent after APTH (123). The primary change was edematous infiltration and hypertrophy of orbital fat. The hypertrophy of orbital muscle was in part secondary to the changes in other tissues (124). These changes closely resembled those found in cases of human post-thyroidectomy exophthalmos (119).

Smelser (122) refers to the fact that exophthalmos due to cyanide can be distinguished from that due to APTH. The former type is preventable or removable by 1) excision of the superior cervical ganglion or 2) by administration of iodine or fruit juice. The latter type is unaltered.

In their work with methyl cyanide Marine and Rosen (75) noticed that along with the development of exophthalmos there were often signs of precocious sexual development, particularly after thyroidectomy. In 1936 they reported (76) that exophthalmos was most easily produced in young male rabbits which were most active sexually. Gonadectomy was performed on 38 young male and 23 young female rabbits. In none of these would cyanide produce exophthalmos and in 3 rabbits which had developed frank exophthalmos the exophthalmos receded after gonadectomy. Other investigations were made at the same time but no final conclusions were drawn. Smelser (124) found that if estradiol was injected into female guinea-pigs their extra-ocular muscles increased in weight.

Thus, in guinea-pigs at least, exophthalmos may be produced by the use of APTH after thyroidectomy, by gonadal substances after thyroidectomy and by administration of cyanides. In cats and dogs exophthalmos has been reported following the administration a) of thyroid extracts with ephedrine or b) of adrenal extracts with a sensitizing substance which has sometimes been thyroid extract.

Thus, apart from stimulation of Müller's orbital membrane through sympathetic nervous paths, adrenalin with thyroid extract or some other sensitizing agent, APTH, cyanides or alterations in the orbital circulation might produce exophthalmos in lower animals. Which of these is the normal method of production? Unfortunately the lower animals seldom show exophthalmos. Where cases of thyrotoxicosis are seen in horses, dogs and other animals veterinary surgeons could do valuable work by recording and publishing the results of their treatment.

#### EXOPHTHALMOS OF ENDOCRINE OR NERVOUS ORIGIN IN HUMAN SUBJECTS

The purpose of this section is to review investigations which have been made on those conditions of exophthalmos in man which appear to be related either to the products of endocrine glands, to the autonomic nerves, to weakness of the extrinsic muscles of the eye or to a combination of these conditions. No further reference will be made to exophthalmos associated with oxycephaly, cavernous sinus thrombosis, high intra-ocular pressure, rat bite fever or tumours of the orbit, nasopharynx or cranial cavity.

The terms 'exophthalmos' and 'proptosis' will be used interchangeably (though

Mann (72) has defined them separately) and without implying any theory of the mechanism which causes the eyeball to advance in the orbital cavity.

In human subjects exophthalmos has been associated with action of the thyroid gland since Graves (41) and Basedow (9) described the disease which bears their names. Enquiry was first directed to the immediate mechanism by which the eyeball was made to advance in the bony orbit. Experiments on lower animals failed to help in this matter since their orbital anatomy is different from that of man. Enquiry also sought to ascertain exactly the chain of stimuli which set in motion the immediate mechanism. This enquiry uncovered the fact that exophthalmos might persist and increase after removal of the thyroid gland and might sometimes even be relieved by administration of thyroid extract. Results of experiments on animals were available at this stage to suggest that this post-thyroidectomy exophthalmos depended on anterior hypophyseal thyrotropin (called APTH in this paper). These experiments do not disprove the existence of exophthalmos due to thyroxin, but no exact explanation of thyrotoxic exophthalmos has so far received universal assent.

In order to produce exophthalmos a change must occur either in the tension of some orbital muscle or in orbital fluid pressures. This change constitutes the immediate mechanism (proximate cause) of exophthalmos. One assumes that this immediate mechanism must, itself, be put in operation by a nervous or chemical stimulus. The stimulus might be a nerve impulse initiated at the cerebral or at a lower level. It might be an endocrine product which could activate or inhibit striped or smooth muscle (including the muscle of blood vessels). It might be something which injured the extra-ocular muscles or their nerve supply.

Evidence relating to orbital structures which have been suggested as possible immediate mechanisms of exophthalmos will first be considered. *Smooth muscle mechanisms:* 1) Müller's orbital muscle; 2) Smooth muscle in the anterior region of the orbit; 3) Smooth muscle in the posterior region of the orbit; 4) Tenon's capsule. *Extra-ocular (striated) muscles:* 5) Muscle weakness with a high BMR; 6) Muscle weakness with a low BMR; 7) Exophthalmic exophthalmoplegia; *Infiltration mechanisms:* 8) Edema of the orbital tissues; 9) Fat infiltration. Following this evidence the influence of endocrine products and of the nervous system will be considered and finally reference will be made to lid retraction which has often been mistaken for true exophthalmos.

#### *Smooth Muscle Mechanisms*

*H. Müller's orbital muscle.* An illustration of this muscle as it occurs in man may be found in Whitnall's *Anatomy of the Human Orbit* (134). After carefully defining its limits Whitnall writes: "It is hardly possible that the contraction of such a small muscular mass . . . could affect the position of the eyeball by compression of the mobile orbital fat." Salomonsohn (113) and Hesser (47) had expressed the same opinion. Kraus (60) had mentioned the suggestion that the muscle might have an effect but said that more evidence was needed before any such statement could be made. The small muscle found in man is very different from the strong muscular cone which exists in lower animals. On this account the results of experiments on the dog or cat cannot be applied to man without reservations (18).

*Smooth muscle in the anterior part of the orbit.* Photomicrographs have been published which show the muscle in the new-born infant (60). They give no support to the suggestion that contraction of the muscle might constrict veins which pass between its fibers towards the sphenopalatine fossa. Kraus mentions the suggestion that the muscle might alter the posterior part of the orbital cavity or the orbital drainage. He adds that it will be the task of experimental and clinical workers to find how far these possibilities are proved to exist. Hesser (47) published photomicrographs of the orbital contents made in many different planes in the orbits of dissecting-room subjects. He declared that Müller's orbital muscle could not produce exophthalmos and that no evidence was known to him which would enable him to decide what was the immediate mechanism.

Cooper (25), in discussing 5 cases of exophthalmos with tachycardia, argued well against the suggestion that the exophthalmos was due to high intraocular pressure. He suggested as cause a morbid excitation of the levator palpebrae superioris which, he said, was "not uncommon in nervous and hysterical females." This increased levator tone has been recognized since Cooper's time and Birch-Hirschfeld (13) reported that an exophthalmos of 0.5 mm. had been produced by voluntarily raising the upper lid. Elevation of the lid has often been mistaken for exophthalmos. It will be considered below.

Landström (65) described his anterior orbital muscle as passing from the orbital septum to the equator of the globe like a ring having its greatest development on the medial side of the orbit. Sattler (115) replied that the function of this muscle was to draw the conjunctiva back during medial and lateral movements of the eye in the horizontal plane, an opinion also held by Whitnall and Beattie (135). Sattler suggested that if Landström's muscle produced exophthalmos it ought to be innervated by the sympathetic nervous system and that, if so innervated, it ought to contract if injections of adrenalin or cocaine were made into it. He made such injections but no exophthalmos followed. The experiments were not entirely conclusive and Landström's views have been accepted by Mulvany (88) who does not offer any new evidence to support them but illustrates his views in a diagram (fig. 3).

*Smooth muscle in posterior part of orbit.* Hesser (47) published a few photomicrographs from sections made behind the globe. He wrote: "Smooth muscle is found in two positions in the (human) orbit—around the globe and in the inferior orbital fissure. Occasionally definite muscular bundles can, perhaps, be met with also in some other part of the periorbita. I have seen, for instance, an inconsiderable group of a few fibers on the medial wall of the periorbita. But I have never seen smooth muscle on the superior part of the orbit and only in the superior orbital fissure to the same extent as in the inferior orbital fissure." He adds: "I forbear to give a positive opinion on the genesis of this symptom (exophthalmos) since, on the basis of investigations so far completed, it can only be doubtful." D. S. Russell (111) studied serial sections of the whole orbital contents which were situated behind the eyeballs, both in patients who died as the result of accidents and in those of others who died while suffering from Graves' Disease with exophthalmos. She found in all the orbits smooth muscle fibers in the periorbita running roughly parallel to the equator of the eyeball near the postero-lateral wall and the postero-lateral part of



the roof of the orbit. Two of her photomicrographs are reproduced in an article by the writer (18). She found lymphorrhages in the orbits of only one case of fatal Graves' Disease with exophthalmos.

*Tenon's capsule.* Tenon (127) described a fascial capsule covering the straight and oblique extrinsic muscles of the eye. This capsule was attached in front to the bony margin of the orbit and was reflected back on the globe. Sappey (114), Motais (184), Groyer (43a), and Hesser (47) all claimed to find involuntary muscle fibers in the check ligaments (134). Starling (126) stated that the muscle of Tenon's capsule was innervated by the sympathetic nervous system. Morley (83) has stated his views on the production of exophthalmos in Graves' Disease as follows: "The check ligaments are arranged in continuity with the capsule of Tenon. The check ligaments, as Sappey showed long ago, are chiefly composed of smooth muscle fibers and the capsule, though largely fibrous, can be shown by differential staining to contain a considerable proportion of smooth muscle. These structures, the check ligaments and the capsule of Tenon, combine to form a sort of catapult, which, when it contracts, projects the eyeball forwards." The attraction of this explanation depends on a belief that the anterior points of attachment of the check ligaments can really form fixation points for the catapult elastic. Whitnall's (134) description of the attachments and his illustrations on pages 129, 147, 289, and 294, should be compared carefully with the illustrations in Duke-Elder (30) on pages 178-179 and with figure 2 of Mulvany's (88) article on page 599. The position of the equator of the globe relative to the anterior orbital margin may well decide the value of this hypothesis.

It is tempting to imagine that smooth muscle fibers somewhere in the orbit might be made to contract through stimulation of the cervical sympathetic nerve trunk, but stimulation of this trunk fails to produce exophthalmus in human subjects or higher apes, though an analogous procedure will produce exophthalmos in dogs and cats (99).

#### WEAKNESS OF EXTRA-OCULAR MUSCLES

Plummer and Wilder (97) in a noteworthy review distinguished exophthalmos with a high BMR from exophthalmos with a low BMR. This distinction had been revealed by a number of reported cases where 'thyrotoxic' exophthalmos had persisted or increased after thyroidectomy (20, 29, 91, 107, 139). Evidence from animal experiments which has been reviewed above already suggested a connection between exophthalmos with a low BMR and the anterior pituitary body. Other conditions in which exophthalmos may exist with a low BMR include myxedema, exophthalmic ophthalmoplegia, bulbar paralysis and myasthenia.

*Exophthalmos and muscular weakness with high BMR.* Cooper (25) stated that Dalrymple believed that the exophthalmos of Graves' Disease was due to weakness of the extrinsic muscles of the eye. Möbius (80) agreed. Askarazy (2) examined various skeletal muscles from 4 patients who died of Graves' Disease and reported that degenerative changes might be found in any striated muscle except that of the heart. The unbelievers suspected post-mortem changes. When successful thyroidectomies became common, the orbital contents available for examination post-mortem became fewer. L. B. Wilson in a personal communication to Plummer and

Wilder (97) stated that in post-mortem examinations made on 8 patients who died of active Graves' Disease before 1917 he had found little orbital fat or edema but had found small and degenerated muscles in the orbital cavity and also weak muscles in the thigh. Plummer and Wilder's table 7 gave figures relating the degree of exophthalmos to a physiological test for thigh muscle weakness in 1000 cases seen before 1922 and 1000 cases seen after 1930. The authors reported a positive correlation between weakness of the quadriceps extensor muscles and exophthalmos. Weakness of extra-ocular muscles has also been noted by Brain (16) and Mulvany (87, 88). Infiltration of fat has also been found in cases of thyrotoxicosis (109) and will be considered under fat infiltration below. The infiltration of water or fat after thyroidectomy will be considered in relation to anterior pituitary thyrotrophin.

*Exophthalmos and muscular weakness with low BMR.* Exophthalmos appears with a low BMR in the following cases: 1) primary thyrotoxicosis followed by thyroidectomy; 2) primary thyrotoxicosis followed by atrophy of the thyroid gland; 3) those which seem explicable by primary thyroid deficiency and relative overaction of the anterior pituitary; and 4) those which seem explicable by simultaneous thyrotoxicosis and over-production of APTH (72). The essential feature of all these cases is an active anterior pituitary gland which alters the condition of the orbital muscles and connective tissues in much the same way as it has been shown to do in thyroidectomized guinea-pigs (120). The condition of the muscle in post-operative cases will be described here. The evidence which connects the condition with the pituitary gland will be given later.

Burch (20) reported a case of a male aged 62 who came with 7 mm. exophthalmos 2 years after a sub-total thyroidectomy and with right eye exenterated. The extra-ocular muscles were greatly enlarged and exhibited fiber degeneration, edema and lymphocytic infiltration. Degenerated fibers had been replaced by fibrous tissue. The orbital fat, connective tissue and nerves seemed normal. Burch referred to previous authors who had found some of these conditions in goitre cases. Naffziger (90) examined and operated on the orbits of 6 patients who suffered from severe exophthalmos after removal of their thyroid glands. The orbits showed edema of both the cellulo-adipose tissue and the muscles with some infiltration of small round cells. There was also some edema of the lids and conjunctiva with lacrimation and impairment of the movements of the eyeball. Swelling of the optic disc was present in 5 of the cases. Orbital decompression was performed through the orbital roof. Some connective tissue and fat were removed from the posterior part of the orbit as well as portions of extrinsic muscles. The muscles were swollen so as to reach 3 to 8 times their normal size. Microscopical examination of them revealed edema, loss of striation, collagen in interfibrillar spaces and collections of small round cells the size of lymphocytes near the blood vessels. The operation reduced the exophthalmos by amounts which varied from 2 to 7 degrees, presumably 2 to 7 mm., on the exophthalmometer. It relieved the edema of the orbit and the aching sensation which 3 of the patients had felt and had localized behind the eyes. The exophthalmos did not recur. In the discussion on Naffziger's paper Katz (90) described a similar case in which operation had produced 5 mm. reduction in exophthalmos.

Other cases of post-thyroidectomy exophthalmos have been described (37, 45, 87, 88, 92, 107, 108, 139, 140). Thomas and Woods (128) reported on 18 such cases and Marine (73) collected 56 such cases of which 60 per cent were in males. Muscle changes were prominent in nearly all of these cases.

*Exophthalmic ophthalmoplegia.* Under the name of exophthalmic ophthalmoplegia, Brain (16) grouped 31 patients who showed exophthalmos and ophthalmoplegia. In 29 of these cases the exophthalmos was the first cause of complaint; in two cases the exophthalmos began after thyroidectomy. Brain believed that in these cases thyrotoxicosis played no essential part since thyrotoxic symptoms were generally slight or absent and the BMR was normal or subnormal. Treatment by thyroidectomy was ineffective. In distinction to myasthenia gravis the paralysis was usually limited to the ocular muscles. The muscle weakness was not characteristic of myasthenia and was not relieved by administration of physostigmin. The ophthalmoplegia was attributed to the mechanical effect of the intra-orbital tension on the muscles. Brain stated that 1) eye symptoms are usually the first cause of complaint; 2) the average reading of the exophthalmometer tends to be higher than in hyperthyroidism (7 mm. compared to 2 mm.), but the patient's placid appearance is in striking contrast to the anxious expression of the typical patient with exophthalmic goiter; 3) in 13 out of 18 cases the ophthalmoplegia was unilateral and in all these 13 the elevating muscles were weak or paralyzed whereas abduction was only reduced in 4 cases and adduction or depression in one case each; 4) in no case at the time of study was there evidence of any lesion of the central nervous system though in one case there had been generalized muscular wasting which had recovered completely after partial thyroidectomy; 5) cutaneous pigmentation was often noted.

The extra-ocular muscles were only available for examination in two of Brain's cases. In one case (*case 1*) their appearance suggested edema and there were areas of infiltration by leucocytes. The patient's thyroid gland showed the changes characteristic of Graves' Disease. Death had been due to pneumococcal pericarditis. The second case (*case 6*) had previously undergone thyroidectomy. His thyroid when examined after operation suggested thyrotoxicosis. When he was being treated for exophthalmic ophthalmoplegia a fragment of his levator palpebrae muscle was removed at biopsy. Compared with a control muscle prepared similarly for microscopic examination its fibers showed changes in cross striation and staining. The mode of the diameters of 100 fibers was  $25\ \mu$  as compared to a mode of  $15\ \mu$  in the control muscle.

Unfortunately no details were given to show the distribution of signs in the 31 individual cases, but, apart from the two which were post-thyroidectomy cases, the other 29 might have been divided into those with and without signs of thyrotoxicosis such as rapid pulse, moist skin, tremor of hands and high BMR. If this had been done the cases of exophthalmic ophthalmoplegia might all have been classified as either thyrotoxic exophthalmos with raised BMR, post-thyroidectomy exophthalmos, or primary thyrotoxicosis followed by functional decadence of the thyroid gland. The ophthalmoplegia might have been due either to thyrotoxicosis or to the changes in the muscles which occur when APTH is not neutralized by thyroxin.

Brain's statements, often modified, have been put inside quotation marks by

Mulvany (88) and have been vigorously criticized. Those who are interested should read the papers for themselves. Mulvany considered that "the term exophthalmic ophthalmoplegia . . . could appropriately be dropped for the more accurate designation of its two components; thyrotoxic ophthalmoplegia and thyrotrophic ophthalmoplegia" (88). The cases which he gives as examples of these two types of ophthalmoplegia might serve as examples of Plummer and Wilder's classification of exophthalmos with high or low BMR.

### *Infiltration Mechanisms*

*Edema of the orbital tissues.* The title given to Basedow's (9) classical article of 1840 was "Exophthalmos Due to Hypertrophy of the Connective Tissue of the Orbit". The following passages are translated from it: "I have already given my opinion on the immediate cause of the exophthalmos that it is a strumous hypertrophy of the cellular tissue behind the globe, in the words of St. Yves 'un amas d'humeurs qui se font derrière la globe de l'oeil'. Yet I hold that this hypertrophy is a secondary sign of a diseased circulation and of a faulty crasis (*sic*) of the blood, a dyscrasia which, by means of the scrophule already described, appears in diseased vegetative glands and their tissue". In Basedow's time the coagulating property of the blood was considered to be of importance for general health and references to this may be found in contemporary English medical literature. Microscopical examinations were not possible in Basedow's cases and Basedow does not pretend to know which cell constituents of the connective tissue, if any, hypertrophy.

In lower animals exophthalmos has been produced experimentally by producing an imbalance between the blood supply to the orbital cavity and the drainage of the cavity. This imbalance may be due to a rise of arterial blood pressure (19), to interference with the venous outflow (69) or to dilatation of the orbital arteries by acetyl choline (19). High arterial blood pressure is not noticeable in Graves' Disease and patients suffering from high blood pressure do not usually exhibit exophthalmos so it is not likely to be responsible for the exophthalmos. By obstruction of the anterior orbital vein when the subject's head was upright, Birch-Hirshfeld (13) produced 1 mm. of proptosis. Obstruction of orbital veins has never been shown in Graves' Disease but its occurrence has not been disproved. Basedow (9) and Trousseau (129) certainly believed that the blood supply to the orbit was increased in Graves' Disease. Fuchs (38) suggested dilatation of the carotid arterial tree. Arterial dilatation seems at first thought improbable in a state which includes some stimulation of the sympathetic nervous system (the nerve to the superior tarsal muscle) but circulatory imbalance may possibly be a factor in the exophthalmos of Graves' Disease. It almost certainly occurs in post-thyroidectomy exophthalmos as a result of muscle hypertrophy and venous obstruction.

*Fat infiltration.* The orbits examined by Naffziger came from cases of exophthalmos which recurred or increased after thyroidectomy. The chief changes seen in them were increase in the size of the extra-ocular muscles, lymphocytes and the edema which has been mentioned. Smelser (120) obtained very similar results of guinea-pigs which were treated with APTH after thyroidectomy but he mentions solid masses of fat fairly free from ordinary connective tissue. Paulson (95) confirms this

work on guinea-pigs. Deposition of fat after thyroidectomy seemed possible but it was with surprise that the writer learned that deposition of fat also occurs in thyrotoxic cases. Rundle and Pochin (109) described 17 cases where the patient died while suffering from Graves' Disease with or without some exophthalmos. The extrinsic muscles, lacrimal glands and residual tissues were weighed separately, dried at 108°F. to constant weight and then extracted with chloroform or ether in a soxhlet apparatus. The water content expressed as a percentage of the fat-free weight of the structure was practically identical in the muscles and lachrymal glands of normal and thyrotoxic subjects. It was slightly *lower* in the 'residual tissue' of the thyrotoxic cases than in those of the normal cases. In this respect the results were different from those of Smelser (120) when APTH was given to thyroidectomized animals. Rundle and Pochin report that "exophthalmos in Graves' Disease is accounted for quantitatively by an increase in bulk of the retro-bulbar tissues. The increase is relatively greatest in the eye muscles of which the average fat content was doubled in a series of 17 thyrotoxic cases. . . . Increase of fat in the orbital fatty tissue is, however, responsible for *most* of the increase in bulk". Control orbits from obese patients showed no increase of orbital fat. The authors are to be congratulated on obtaining for study so many suitable orbits. Rundle and Wilson (110) studied further the bulging of eyelids with exophthalmos and showed that prolapsing orbital fat as well as edema could cause the sign.

Increased fat content had previously been offered as an explanation of exophthalmos in Graves' Disease by Askarazy (2), Bristowe (17), Mendel (78), Murray (89) and Mackinnon (70). Each of the last two authors described a single case of Graves' Disease in which exophthalmos was accompanied by increased orbital fat. In Murray's case one of the orbits contained half an ounce (14 gm.) of fat. Neither of these cases was seen at the initial stage of exophthalmos. Cases of Graves' Disease have also been examined in which no abnormal fat could be found (51, 93, 111). This fact makes it difficult to accept Smelser's suggestion (120) that both hyper- and hypothyroid exophthalmos are produced in the same way.

#### ENDOCRINE PRODUCTS AND EXOPHTHALMOS

For the study of endocrine extracts the dog, cat, guinea-pig and rabbit were used before human subjects. Owing to anatomical differences between human and sub-human orbits, results based on the latter may not always apply to man (18). Further, in observing patients investigators did not usually distinguish between true exophthalmos and lid retraction (Dalrymple's sign) in Graves' Disease. Lid retraction often gives the impression of exophthalmos when under observation or in full-face photographs. This has been recognized by many thoughtful observers of Mr. Eddie Cantor and of the British actors Mr. George Robey and Mr. Leslie Henson. Fewer people have recognized that even photographs taken from the side (at right angles to the face) can suggest exophthalmos when only lid retraction is present. The method of Birch-Hirshfeld (13) cannot be used for hospital cases as it demands the accurate fitting of a lined metal helmet to each subject's head and a rigid fitting of the camera to the helmet. Exophthalmometers have been described by Hertel (46) and Pochin (99). With these a series of readings can be made from a

base line of the lower orbital rim or the outer orbital rim. Plummer and Wilder (97) noted cases in which "one eye seems to be more prominent than the other, but the exophthalmometer readings are identical and the apparent prominence is entirely due to the retraction of the lids". Measurement of the lid retraction has been studied by Pochin (98, 100) and will be considered later.

*Thyroid extracts.* The earliest experiments with thyroid extracts were made by patients who took them in order to reduce their weight. Moorhead (82) described the case of a woman who had taken thyroid extract for over two years and came to an oculist suffering from exophthalmos and thyrotoxicosis. Justin-Besançon and others (53) quote similar cases of Bécclère and Rist. Brain (15) described the case of a woman who produced unilateral exophthalmos by taking thyroid extract over a long period and whose condition was cured by ceasing to take it. In Brain's case the exophthalmos was genuine and not merely lid retraction. Brain could only find in the literature about 20 cases where the exophthalmos was stated to be due to thyroid extract and some writers have denied that thyroid extract can produce exophthalmos. They have been upheld in their denial by cases of Graves' Disease in which no exophthalmos is present and other cases where thyroidectomy has failed to relieve the exophthalmos though the heart and metabolic rates fell after the operation. Seitchik (116) mentions 11 cases of exophthalmos which followed the administration of thyroid extract, taken in 4 cases for the relief of obesity and in 7 cases for the relief of myxedema. Plummer and Wilder (97) published figures to show that the frequency of exophthalmos in 1000 cases before 1922 and in another series of 1000 cases seen after 1930 varied with the BMR and so, presumably, with the activity of the thyroid gland. They agree that quite exceptional cases occur. Case 5 described by Brain (16) had been treated by partial thyroidectomy for chronic thyrotoxic myopathy. "The body musculature recovered completely after a partial thyroidectomy", he stated. The increase of fat which Rundle and Pochin (109) measured in the orbits of patients who had died while suffering from Graves' Disease is further evidence of a connection between thyroxin and increased fat content of the orbit. This increase of fat has not been reported in cases of post-thyroidectomy exophthalmos, so far as the writer knows.

*Thyroxin and adrenalin.* An article by Sainton and Hesse (112) describes the production of exophthalmos and other symptoms of Graves' Disease in a myxedematous woman aged 38 by a rather strenuous treatment with thyroxin and adrenalin. The article is illustrated by full-face photographs only, but as pseudo-exophthalmos was recognized by workers in Paris at that date, one may assume that the exophthalmos was a true one. Justin-Besançon and others (53) describe the temporary appearance of the exophthalmos in a patient with Graves' Disease but normal eyes when ephedrine was administered. (Ephedrine delays the inactivation of adrenalin (39) as physostigmin delays the inactivation of acetyl choline.) Urechia and Retezeanu (130) reported the temporary production of moderate exophthalmos when thyroid and ephetonine were given at the same time to a patient.

One might distinguish the causation of these types of exophthalmos by naming them 'thyrotoxic exophthalmos' (88) in the patients who took thyroid extract only, 'thyro-adrenalin exophthalmos' in Sainton and Hesse's case and 'thyro-ephedrine

exophthalmos' in the cases of Justin-Besançon and Urechia and Retezeanu. Brain's case (15) might perhaps be included as a case of thyro-adrenal exophthalmos since facial hair suggested hyperactivity of the adrenal cortex.

*Anterior pituitary gland extracts.* Anterior pituitary gland extracts were first used to produce exophthalmos in rabbits and guinea-pigs (35, 68, 75, 104). Smelser (120) published photomicrographs to illustrate the similarity between orbital tissues in thyroidectomized guinea-pigs so treated and the orbits of patients who had exophthalmos after thyroidectomies. Both in guinea-pigs and in human subjects there was edema in the connective tissues and between the muscle fibers. More degeneration of muscle fibers was present in the guinea-pig than in the human orbits.

Neither Smelser nor Pochin (101) who also used guinea-pigs reported any increase of orbital fat as the result of APTH. In human subjects Mulvany (87, 88) contrasted 'thyrotoxic' and 'thyrotrophic' exophthalmos, associated respectively with thyrotoxicosis and excess of APTH. In the latter condition "the orbital fat is little disturbed except in the presence of a generalized edema when it may become infiltrated with round cells". In the former condition the orbital fat is "normal or slightly increased". Mulvany prepared a table of other differences in the two types of exophthalmos. Beside his suggested name of 'thyrotrophic exophthalmos' the condition has been called 'thyrotopic exophthalmos' by Zondek and Ticho (141), 'paradoxical exophthalmos', 'pituitary exophthalmos' (141) and 'low metabolism exophthalmos' (97). Two pieces of evidence seem to support directly the relation of the exophthalmos to the pituitary gland. Mandeville (71) has reported the relief of post-thyroidectomy exophthalmos by irradiation of the hypophysis. The eyeballs receded 5 mm. He quotes good results reported by Ruedemann (107) and by Thomas and Woods (128) but disappointing results reported by Merrill and Oaks (79) and by Naffziger (90). A crucial experiment was described by Stallard (125). A male patient aged 31 suffered from exophthalmic ophthalmoplegia with a low basal metabolic rate. He was given APTH as a diagnostic test. His BMR rose from about normal to about plus 40 per cent; he showed the signs of Graves' Disease; the exophthalmos increased; a corneal ulcer which resisted treatment perforated and it was necessary to remove the eyeball. The extra-ocular muscles were from 4 to 5 times their normal size. Part of a muscle was removed and showed chronic inflammation with small round cells and interstitial fibrosis but no definite degeneration of the muscle fibers. The action of APTH would seem clear in this case but one point remains unexplained: if it is true that an increased amount of APTH circulates in the blood in myxedema (141), why do not patients with myxedema suffer from exophthalmos? Is it due to lack of adrenal products?

Mann (72) has mentioned cases beside those of post-thyroidectomy exophthalmos which seem to be related to the pituitary body. In 4 cases the symptoms suggested primary thyrotoxicosis followed by atrophy of the thyroid gland. In another 4 cases there was primary thyroid deficiency with excess APTH. All these 4 patients had sallow, thick coarse skin suggesting myxedema. In three of the cases the lids would not close completely but this was due to their swelling and stiffness rather than to retraction. The BMR was measured in three cases and was found to be -9 per cent, -27 per cent and about -1 per cent respectively. Three cases showed proptosis.

Treatment by tarsorrhaphy, excision of conjunctiva and thyroid extract saved the remaining eyes. (Stallard's case (125) may have been an early case of primary thyroid deficiency.) Finally Mann mentions four cases showing loss of weight, tachycardia, proptosis and ophthalmoplegia co-existent. In three cases the BMR's were respectively +38 per cent, +42 per cent and +43 per cent; in the fourth case the BMR was not measured before treatment. These were thought to be due to a combination of thyrotoxicosis and excess of APTH.

*Gonadal products.* Certain gonadal products have been shown to increase exophthalmos in animal experiments but up till now the writer has seen no reports of their action in cases of human exophthalmos.

#### MOTONEURONES IN GRAVES' DISEASE WITH EXOPHTHALMOS

The appearance of exophthalmos both as part of Graves' Disease and after thyroidectomy has often been related in time to emotional shock. (Sollwig quoted Edmunds, 32; Geyer quoted Voss, 131.) In most of the cases which have been reported careful distinction was not made between true exophthalmos and retraction of the upper eyelid but in some cases the exophthalmos was certainly genuine.

It has been natural to seek to explain exophthalmos by the sympathetic nervous system which supplies the smooth part of the levator palpebrae superioris (the superior tarsal muscle). Yet as we have said above stimulation of the cervical sympathetic nerve trunk does not produce exophthalmos in man. The possibility of paralysis following injury to the nerve supply of the extrinsic muscles of the eye was mentioned by Ballet (5) who quoted the opinion of Sattler (115) and others that the exophthalmos of Graves' Disease was due to 'functional disease of the bulb'. Greenfield (43) reported changes in the cervical sympathetic ganglia. L. B. Wilson (136) found hyperpigmentation, granular degeneration, atrophy and reduction of ganglion cells varying with the duration and remissions of the disease in the ganglia of 12 patients who died of Graves' Disease. He produced similar cell pictures by inoculation of bacteria into or frequent stimulation of the superior cervical ganglion (137). Later he referred to examinations made before 1917 on the eye muscles and expressed the tentative opinion that the exophthalmos could be explained by weakness of the recti muscles (138). Heuer (48) thought that the eye-muscle palsies in cases of exophthalmic goiter might be due to cerebral disturbances but had no material for examination. In one such case Wedd and Permar (132) made serial sections from 25 areas between the basal ganglia and the medulla without finding anything abnormal. Risak (105) reported a case where all the symptoms of Basedow's Disease followed an attack of encephalitis lethargica. Penfield (96) reported a case in which exophthalmos and other symptoms followed a diencephalic tumor close to the area of the hypothalamus delimited by Bard. During the attacks the eyeballs were said to protrude. This protrusion, if actual, may have been due to increased intracranial pressure, to nerve injuries which removed controlling impulses from lower nerve paths or to the removal of inhibitions from the pituitary body and, in consequence, an increased production of APTH.

Apart from possible nerve injuries Brain (16) refers to thyrotoxic myopathies: acute, chronic and periodic as well as to myasthenia gravis. Brain's case 5 appears



to have been a case of general thyrotoxic myopathy followed by post-thyroidectomy exophthalmos. The myopathies are distinguished from exophthalmic ophthalmoplegia by their cure if thyroidectomy is possible. Ophthalmoplegia with Graves' Disease may respond to treatment with physostigmin (in which case Brain considers it to be due to myasthenia) or may fail to do so either because it has a different cause or, in some cases, because the treatment is given too late. Cases of myasthenia with exophthalmos have been recorded by L. B. Cox (26), by H. Zondek (140) and by Laurent (66). Rundle and Pochin (109) say that "it appears probable that lid retraction, ophthalmoplegia and possibly other eye signs may prove to be related in some way to the extensive increase in fat content of the extrinsic muscles in thyrotoxicosis".

#### LID RETRACTION

Before summing up the work which has been done on exophthalmos, reference must be made to studies on lid retraction. Both upper and lower lids possess smooth retractor muscles which were described as Müller's palpebral muscles to distinguish them from his orbital muscle. Müller's palpebral muscle of the upper lid is also named the smooth part of the levator palpebrae superioris or the superior tarsal muscle. Pochin (100) describes its origin from the striated fibers of the Levator and its passage forwards to be inserted into the upper border of the tarsal plate. This plate in turn is attached to the skin of the lid at its free lower border, the anterior surface of the tarsus being unattached to the super-jacent skin. Into this part of the skin is inserted the tendon of the striated Levator palpebrae. The superior tarsal muscle is innervated through the cervical sympathetic nerve trunk. In Graves' Disease lid retraction due to it alone may occur without exophthalmos, or exophthalmos may occur without lid retraction (98, 100). At death lid spasm disappears but exophthalmos does not (109). Sympathectomy improves lid retraction but not exophthalmos (117). After sympathectomy voluntary lid retraction can still be performed (100). In Horner's (50) Syndrome as originally described enophthalmos was unimportant but ptosis was definite (99). Electrical stimulation of the cervical sympathetic trunk in human subjects produces lid retraction but not exophthalmos (99). Eden and Trotter (31) noted the presence of lid retraction in 52 per cent of 134 cases of toxic diffuse goiter. The appearance of exophthalmos so produced has already been emphasized. Leriche and Fontaine (67) believed that lid retraction might be stimulated not only by fibers in the cervical sympathetic trunk but also by others which were believed to travel along the course of the vertebral artery. They also described interesting symptoms produced by a neuroma of the cervical sympathetic nerve trunk after removal of the superior cervical ganglion. Mulvany (88) included lid retraction among the signs of thyrotoxicosis and stated that it was absent in uncomplicated thyrotropic exophthalmos. Rundle and Pochin (109) drew attention to the fact that in Graves' Disease the fatty deposits were relatively greater in the levator palpebrae superioris muscle than in other extra-ocular muscles and suggest that 'lid retraction, ophthalmoplegia and possibly other eye signs may prove to be related to the extensive increase in fat content of the extrinsic muscles in thyrotoxicosis.' The same authors have drawn attention to the fact

that bulging of the eyelids in Graves' Disease may be due to overfilling of the orbital cavity so that the orbital fat comes forward as it does also when tumors are present in the orbit. They distinguish this fatty swelling from edema of the lids (110).

#### SUMMARY

In lower animals exophthalmos may be produced experimentally by stimulation of the cervical sympathetic nerve trunk, by administration of thyroxin together with certain sympathomimetic substances or by the use of cyanides. These act by causing contraction of Müller's orbital muscle which, in turn, increases the fluid tension behind the eyeball and drives it forward. Almost the whole of the nervous path is known from the hypothalamic region to Müller's orbital muscle. In apes, stimulation of the cervical sympathetic trunk fails to produce exophthalmos. Exophthalmos may also be produced in the absence of the thyroid gland by anterior pituitary thyrotropic hormone (APTH). It is associated with edema of the orbital contents. The effect of APTH is increased by gonadal substances.

True exophthalmos in Graves' Disease must be distinguished from lid retraction by means of an exophthalmometer. It may exist with a high or with a low BMR. Evidence shows that the form with a high BMR is accompanied by considerable increase of orbital fatty tissue and increase of fat in the extra-ocular muscles. Evidence suggests that this form may be produced by the administration of thyroxin (82, 15). It is usually but not always accompanied by lid retraction. It has been produced in a hypothyroid patient by administration of thyroxin and adrenalin (112) as well as by thyroxin and ephedrine or ephedrine (54, 130). Other possible but unproven factors in its causation are smooth muscle in the orbit or disturbances of the orbital circulation. Müller's orbital muscle is not responsible for exophthalmos in Graves' Disease.

Exophthalmos with a low BMR (hypothyroid, post-thyroidectomy, thyrotropic exophthalmos) is due to the action of an anterior pituitary substance (APTH) which produces edema and round cell deposits in the loose orbital tissue and in the extrinsic muscles, sometimes increasing their volume to such an extent that not only is the eyeball pushed forward but the circulation in the orbit is obstructed. It is uncertain whether this muscle change is primary or follows changes in the orbital connective tissues as it did in Smelser's guinea pigs.

Brief consideration has been given to the occurrence of exophthalmos due to ophthalmoplegia and myasthenia. Treatment of the condition has not been considered.

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## ADRENAL CORTEX AND WATER METABOLISM<sup>1,2</sup>

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THE ROLE OF THE ADRENAL CORTEX in electrolyte and water metabolism was the first of the important adrenal cortical functions clearly recognized when studies of cortical physiology began to take precise form in the early 1930's. Emphasis has been placed, however, upon the actions of cortical hormones on electrolyte metabolism, to which their actions on body water were generally considered as a secondary osmotic consequence. In particular, the fact that the cortical hormones cause sodium retention, and hence water retention, led to the idea that their major action on water metabolism was one of water conservation.

Within recent years, on the other hand, it has become clear that a definite function of cortical hormones is to stimulate water diuresis. In a water-loaded animal this action can be a dramatic life-saving one. Under proper circumstances the water-eliminating effect of cortical hormones is of comparable magnitude to that of the water-conserving action of their physiological counterpart, the posterior pituitary antidiuretic hormone.

The dilemma which presents itself is, therefore, that the cortical hormones may cause either water retention or water loss. This is not an unusual situation because many substances exert different effects depending upon the setting of the physiological stage on which they operate. Evidence now at hand is sufficient to permit an intelligible if not necessarily complete interpretation of these different aspects of adrenal cortical function.

### I. ADRENAL CORTEX AND ELECTROLYTE METABOLISM

No attempt will be made here to review comprehensively the extensive literature on the effect of the adrenal hormones on electrolyte metabolism, since that has recently been done (241, 95, 122). A summary will be given, however, of data basic to subsequent considerations.

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<sup>2</sup> Abbreviations used in this review: ADH = posterior pituitary antidiuretic hormone; DCA = desoxycorticosterone acetate; ACTH = adrenocorticotrophic hormone of the anterior pituitary.



While considerable earlier information existed (71), the reports of Loeb *et al.* (134) and Harrop *et al.* (93, 91) in 1933 gave convincing demonstration of the fact that adrenal insufficiency was characterized by renal loss of sodium salts. The work of many investigators provided the details showing that a renal wastage of sodium, chloride and bicarbonate characterize adrenal insufficiency in most animals and in man although species differences (223, 215, 21) may exist. This loss was sufficient to explain some, but not all, aspects of the adrenal insufficiency syndrome and in addition provided a rational basis for the demonstration of the therapeutic benefits of salt feeding in adrenal insufficiency (231, 133, 135, 186) and increased salt appetite after adrenalectomy (178, 180). Of the known cortical steroids DCA repairs the sodium deficiency most effectively and when given in overdosage will cause excess retention of this ion (92, 241). An action of exceedingly small quantities of DCA can be detected on the excretion of radioactive sodium (48). It has also been found that the sodium and chloride content of sweat is high in Addisonian patients and in normal individuals is reduced by either DCA or ACTH (39).

The possibility is subsequently discussed that some of these apparent actions of the cortical hormones on NaCl metabolism are directly due to an imbalance between adrenal cortical and posterior pituitary activity.

When it became evident that alterations in sodium metabolism would not explain all the phenomena seen in adrenal insufficiency (237), attention was focused on potassium which may increase both in the serum and tissues after adrenal removal (241, 122). The adrenalectomized animal is highly sensitive to the toxic effects of high serum levels of potassium, as it is to other deviations from a normal internal or external environment, and in certain instances potassium accumulation presumably contributes to death in adrenal deficiency. Again DCA most effectively stimulates the excretion of potassium and given in excess may reduce plasma potassium to a dangerous level in the dog (125), man (255) and probably in the rat (81). Such reduction is accompanied by sodium retention and by the replacement of cellular potassium by sodium (55). These findings together explain well the fact that the best maintenance of adrenalectomized animals, aside from hormone replacement therapy, is to provide a high sodium-low potassium diet (2).

It is generally accepted that the kidney tubule is the primary site of action of the corticoids as far as their effects on sodium and potassium excretion are concerned. They facilitate the reabsorption of sodium and inhibit the reabsorption of potassium (89, 241, 183); the opposite occurs in adrenal insufficiency. The curious observation has been made (183), however, that when high plasma sodium levels are maintained in adrenalectomized dogs by infusion, the reabsorption of sodium is increased above normal—just the opposite of what occurs when plasma sodium is within normal or sub-normal ranges.

Certain of the gonadal steroids simulate the action of DCA in causing sodium retention (252), but judging by earlier experiments all cortical steroids do not. In acute experiments some corticoids, e.g., corticosterone and dehydrocorticosterone (250, 35), are weak sodium retainers and some, 17-hydroxycorticosterone and 11-dehydro-17-hydroxycorticosterone (compound E), enhance sodium excretion (251, 225a). Chronic experiments revealed that the latter effect is a transitory one (114, 225a).

Recent work shows that generalization on the specific action of any cortical steroid is very difficult. Forsham *et al.* (60), for instance, have found that compound E, which under some conditions induced sodium excretion, would cause mild sodium retention in Addisonian patients; this effect was antagonized to a certain extent by the addition of DCA! On the other hand, ACTH returned toward normal the altered sodium and potassium plasma levels induced by DCA in normal rats (279). Further confusion is provided by the demonstration that chronic use of DCA may cause in certain conditions a negative sodium and potassium balance with elevated water exchange (152, 283).

It is obvious that the over-all effects of the secretions of a normal adrenal on electrolyte metabolism may be different and hardly predictable from the actions of individual steroids. The action of a given steroid is dependent upon dosage, duration of treatment, other steroids present and additional less well defined physiological variables. As will be noted later, however, all adrenal preparations that have been studied stimulate water diuresis in normal and water-loaded animals.

## II. EFFECT OF ADRENAL CORTICAL HORMONES ON WATER EXCRETION

Rowntree and Snell (185), from observations of human patients, were the first to call attention to the fact that water was not excreted normally when given to an Addisonian subject. Subsequent isolated observations were made by several workers and more recently extensive studies on the subject have been made both in laboratories and clinics.

*Response to Fluid Administration in Adrenalectomized Animals and Addisonian Patients.* Just as is true of ADH, the actions of cortical hormones on water excretion are best seen if observed in an animal or patient in a hydrated state. Regulatory mechanisms for eliminating excess water are then brought into play and if deficient are readily detected.

Animal experimentation on the subject was initiated by Silvette and Britton (214) who showed that adrenalectomized cats excreted either water, urea solution, or salt solution, given intraperitoneally, at a markedly delayed rate. The inference was drawn that the effects were of extra-renal origin and due to some avidity of the tissues for water.

Rigler (181) showed that the administration of water, glucose solution, hyper- or hypotonic NaCl solution to adrenalectomized mice failed to elicit the expected diuresis. Swingle *et al.* (238) obtained similar results with water administration in adrenalectomized dogs, as did Gaunt, Remington and Schweizer (77) in both adrenalectomized and hypophysectomized rats. All of these workers noted, in addition, a marked susceptibility to water intoxication after adrenalectomy, a susceptibility later shown to be in part due to extra-renal factors (Sec. VI).

In subsequent work the essential fact that animals or men with either a marked or latent adrenal insufficiency cannot excrete water at a normal rate has been abundantly confirmed (140, 142, 124, 216, 278, 196, 123, 211, 182, 100, 108, 213, 230, 82, 183, 136, 242) and details of the phenomenon elaborated.

The most thorough studies have been made in the rat. At 18 hours after adrenalectomy, this species will excrete a dose of water used conventionally in diuretic

experiments (approximately 4-5 cc/100 gm.) at a normal average rate, but the variability between individuals will be considerably greater than in normal animals (69). If the water load is increased by giving 2 to 5 such doses of water at hourly intervals, a marked deficiency in diuresis is evident and symptoms of water intoxication, not seen in normal animals, intervene (68). If tested 3 to 7 days after adrenalectomy an inability to excrete even the mild single hydrating dose of water is readily apparent (69). This might not be surprising if adrenal insufficiency with its attendant circulatory and metabolic dysfunctions were developing spontaneously during the post-adrenalectomy interval, but with rare exceptions (123) the phenomenon is almost equally marked in those animals maintained from the time of operation in what would usually be considered a 'normal' state by salt, DCA or adrenal cortical extract (68; see also Sec. VII).

Details have been worked out less completely in species other than the rat, but such information as is available indicates that the same pattern holds for the mouse (181, 111), dog (238, 242), opossum (216), hamster (49) and man (172, 182). In the human species there are only a few (128) recorded exceptions to the rule that Addisonian patients fail to excrete ingested water or injected glucose or saline solution normally.

It has also been found that influences which normally augment the diuretic response to ingested water are either ineffective or of reduced effectiveness in the absence of cortical hormones. This is true in experimental hyperthyroidism (70), in the augmentation of diuresis that follows adaptation to excess water (130) and after the administration of epinephrine (73) or KCl (140). A deficiency of cortical hormones may contribute to the appearance of experimentally-induced edema (107), and injection of these hormones into rats will relieve the deficiencies in diuresis and susceptibility to water intoxication seen in hypophysectomy (119) and in both panthothenic acid and riboflavin deficiency (74).

*Effects of Cortical Hormones on the Diuretic Response to Water in Normal Animals.* When diuresis is induced in normal rats by a single dose of water given by stomach tube, its rate of elimination is enhanced by the administration of either DCA (205, 158) or adrenal cortical extract (158). The same is true when cortical extract is used in the opossum (217). Similar results have been seen following chronic treatment with DCA in the dog (148), but this was in the case of animals developing a diabetes insipidus-like state. Such effects are much more clearly seen if water is given in multiple doses adequate to produce water intoxication. Under these conditions, diuresis is markedly stimulated by cortical hormones and it is exceedingly difficult to induce lethal water intoxication (66, 78). In intact but not in the adrenalectomized animals this protection afforded against water intoxication is apparently due largely to effects of cortical hormones on the kidney; after adrenalectomy extra-renal factors can also be detected (Sec. VI). The stimulation of water diuresis and the protection against water intoxication provides one of the most easily demonstrated overdosage actions of the adrenal cortical hormones, and in severe water intoxication also provides one of the few definite demonstrations of a life-maintaining action of these hormones against lethal stress in normal animals.

In the frog, a form in which the response to adrenalectomy and adrenal cortical

hormones is aberrant and ill-understood in other respects (241), cortical hormones cause a retention, rather than elimination, of excess injected water (109).

*Daily Water Exchange in Adrenalectomized Animals and Effects of Replacement Therapy.* The facts outlined above show deficiencies in the excretion of water forcibly administered to adrenalectomized animals. This type of observation has to be correlated with the fact that in the spontaneous daily water exchange, slightly increased urine volume is a characteristic but not invariable symptom of the early and mild stages of adrenal insufficiency in the untreated dog (134, 93, 240). In the rat urine volume may vary from polyuric to oliguric levels after adrenalectomy, depending on the severity of symptoms and other variables not clearly defined (9, 187, 191, 76, 41). Water intake changes less than urine output, resulting in some cases in a reduced water intake/urine volume ratio. This early diuresis following adrenalectomy is not seen in the cat (270) and as adrenal insufficiency becomes severe, oliguria generally supervenes in all species. The post-adrenalectomy water diuresis is associated with and presumably due to the well-known sodium diuresis that follows loss of adrenal function (144a; Sec. I).

It is clear that the kidney can excrete water in amounts somewhat above normal without cortical hormones, but it cannot excrete fluid at anything approaching *normal maximal* rates, when there is need to do so, as after forcing fluid. It has, in other words, lost its adaptability (172) not only for handling water in excess but in dehydration (124) as well.

When dogs are revived from crises of adrenal insufficiency, there occurs promptly a diuresis of considerable magnitude relative to fluid intake (240, 90). This is presumably associated with a return of blood volume to normal and elimination of accumulated metabolites. When adrenalectomized rats are maintained on NaCl therapy, there is an increased water exchange (76, 99, 41) of similar magnitude to that which follows the same treatment in normal animals. Replacement therapy with cortical extract may result in an essentially normal or slightly elevated water exchange (76), the details of the response probably being dependent on the effective hormone dosage used. DCA therapy produces an increased water exchange after adrenalectomy (41). When DCA and salt therapy are combined a high water intake and output results which is much greater than with either treatment alone (41, 99).

*Effects of Cortical Hormones on Daily Water Exchange of Normal Animals.* In proper experimental circumstances, DCA can produce a polydipsia and polyuria in normal animals. This effect, however, varies somewhat in different species and is dependent on the salt intake in some way only vaguely understood. In rats on a normal or low salt intake, DCA has been reported to increase water intake and output by some workers (205, 276, 25, 99, 41, 209, 6) and not to affect it by others (171, 174). All workers who have looked for it, however, have seen tremendous water exchanges in rats given DCA plus a high NaCl intake (41, 171, 99, 25, 174, 276, 209, 81). The original work on the subject was done by Kuhlman *et al.* (125) in the dog where moderate doses of DCA induce a diabetes insipidus-like condition which is also dependent to a considerable extent on the salt intake (171, 148). Confirmation of the work on dogs (272, 147) and demonstration of a similar diabetes insipidus-like

condition following DCA therapy in a human case of myasthenia gravis has been recorded (147). Interpretation is tending toward the idea that the polydipsia rather than the polyuria is primary (171, 148, 174, 160, 3). If so, the DCA-induced condition differs fundamentally from true diabetes insipidus. As discussed later, however, direct effects of DCA on the renal tubular reabsorption of water are probably also involved. In cats, on the other hand, DCA will not affect the water exchange either in the normal or diabetes insipidus animal (272).

The fact that DCA will reduce the intake of NaCl solution after adrenalectomy in self-selection experiments (180) and increase it in normal animals (174) seems paradoxical at the moment.

There is only a single but nevertheless convincing instance known to the authors in which a diabetes insipidus-like condition has been seen following the use of cortical preparations other than DCA. Ingle (113) gave young fifty-gram rats up to 20 cc. per day of adrenal cortical extract in drinking water for 28 days. Growth rate was reduced but no glycosuria appeared. A polydipsia developed exceeding 100 cc. of water intake per day.

In intact dogs given a *constant* fluid and food intake, cortical extract produced variable effects on water excretion while reducing the excretion of sodium and increasing that of potassium (92).

Patients with cardiac disease and associated water retention show a marked diuresis after the administration of large doses of lipo-adrenal extract (132).

*Effects of Cortical Hormones on Water Excretion in Dehydrated Animals.* Results cited above show that the cortical hormones increase diuresis in animals in normal water balance and in those either mildly or heavily hydrated. Adrenal cortical extract will also increase water excretion in rats mildly dehydrated by deprivation of food and water for 20 hours (158). DCA under similar conditions failed to influence urine volume (158) but it may do so in the dehydrated state after it has been chronically administered in the dog (148) or the rat (276).

### III. MECHANISM OF ACTION OF CORTICAL HORMONES ON WATER METABOLISM

*Renal Factors.* The well known alterations in renal function (241, 222, 282, 95, 63, 10, 168, 7, 143, 117) which occur in adrenal insufficiency make it logical to suspect that the kidney itself may be the major site of cortical hormone activity in regulating diuresis. Until recently, however, only fragmentary evidence unsuitable for comprehensive conclusions was available on the point.

Some cases of renal pathology associated with adrenal insufficiency have been described (7, 138, 97, 220). Other workers, however, have reported the absence of such pathological changes or have not observed them frequently enough to explain the consistent alterations in renal function seen in adrenalectomized animals and Addisonian patients (80, 245, 83, 8, 234). In addition, cortical hormones have effects on renal tubules in tissue culture (28).

Decreased clearances of urea, creatinine, and phenolsulphonphthalein have been a long-recognized characteristic of adrenal insufficiency (228, 227, 143, 88, 10, 63, 89). While some studies on adrenalectomized animals (214) and Addisonian patients (245) indicate normal function by ordinary clinical tests for renal disease, more precise observations indicate definite disturbances. Modern methods have shown a

decreased renal plasma flow (245, 264, 62, 266) and glomerular filtration in both Addison's disease and experimental adrenal insufficiency (89, 141, 152, 245, 80, 192, 264, 266, 15); such factors could contribute to a depressed water diuresis. Waterhouse and Keutmann (264) find essentially normal filtration fractions in treated Addisonians despite reduced filtration rates; from this they postulate a reduction in the effective vascular bed. The rate of glomerular filtration will tend to become adapted to varying water loads after adrenalectomy in rats, increasing as larger amounts of water are ingested (15). At any water load, however, the rate of urine flow is depressed more than expected from the decrease in the filtration rate (15). Furthermore, the deficiencies of filtration in adrenal insufficiency can be wholly or partially corrected by either salt (136, 266, 62, 88, 89, 16) or adrenal hormone treatment (141, 152, 245, 264, 89, 16, 192); yet, at least in the absence of heroic therapy, the deficiencies in water excretion remain (136, 68, 142, 16; cf. Secs. VII and XII). The conclusion follows, therefore, that there is an increased tubular reabsorption of water in adrenal insufficiency. The same conclusion was reached by Gersh and Grollman (80) on the basis of cytochemical observations of the kidneys of adrenalectomized rats and Silvette and Britton (217) from inferences on urea excretion. Nelson and Berman (155), studying histological preparations of kidneys from adrenalectomized rats that had been forced into water intoxication in Gaunt's experiments, reported that "the absence of specific glomerular damage and dilation of Henle's loops may indicate that filtration can occur even in those cases where there is practically no urine output."

The accumulation of an antidiuretic substance in the blood of adrenalectomized animals or an increased sensitivity to such substances (13), as subsequently discussed, makes an increased tubular reabsorption of water not only understandable but expected.

The next question is whether an overdosage of cortical steroids will have the opposite action, i.e., whether it will inhibit water reabsorption in the tubules of normal animals. Despite some dissent (193), most direct evidence on the point gives an affirmative answer. Winter and Ingram (272) found that DCA will decrease tubular reabsorption as judged by creatinine clearance both in normal and diabetes insipidus dogs. Similar results were obtained in rats using both DCA and adrenal cortical extract (16) and in a human case given DCA (283). In some circumstances, it increased filtration as well (272, 38, 193). The effect on tubular reabsorption in normal animals is apparently not the result of a reduction in the blood levels of anti-diuretic substances (6), and there is other evidence that the factors operative in depressing diuresis in adrenal insufficiency are not complete counterparts of those that enhance diuresis after administration of cortical steroids in normal animals (12).

Although changes in renal circulation and glomerular filtration may be contributory factors, it seems safe to conclude that the effects of cortical hormones on tubular reabsorption constitute their major action on water excretion. This function cannot be correlated with the renal hypertrophic action of steroids (205, 50) since the substance, testosterone, which most effectively stimulates renal hypertrophy does not induce polyuria (205). Neither will testosterone repair the renal deficiencies in Addisonian patients.

Alterations in the metabolism of kidney slices from adrenalectomized animals

have been reported (44, 253, 254). Russell and Wilhémi (188, 189), for instance, found that kidney slices show both a subnormal oxygen uptake and deamination of amino acids after adrenalectomy and that these functions may be restored to normal by the administration of cortical extract or DCA. The relation of these facts to the excretion of water is obscure. Since the deficiencies in water excretion, unlike those on oxygen uptake and deamination, are very difficult to correct with replacement therapy a functional relation between them is questionable.

It is possible that the hormones which regulate diuresis could act by altering patterns of renal blood flow according to the scheme of Truetta and co-workers (257) but evidence on the point is lacking.

*Relation between Water and Electrolyte Metabolism as Affected by Cortical Hormones.* The knowledge that some cortical hormones cause sodium retention, and thus indirectly water retention, has to be correlated in some way with the fact that these same hormones are diuretics and essential for the process of normal water excretion. The possible relation, therefore, of the diuretic action of cortical hormones to electrolyte metabolism becomes one of great interest.

Many workers have agreed that, *a*) there is a failure of the diuretic response to force-fed water in adrenal insufficiency regardless of whether the salt loads of the body are high or low, *b*) that salt solutions are excreted only little if any better than pure water in the absence of cortical hormones, and *c*) that the action of cortical hormones on water excretion in adrenalectomized and normal animals is not necessarily associated with any apparent causal alteration in salt excretion (181, 172, 140, 142, 214, 66, 68, 82, 242). The same things are true in hypophysectomized animals (119). Furthermore, DCA, the adrenal steroid most effective in causing salt retention, is still diuretic in its action under most circumstances (Sec. II). From this it might be concluded that the cortical hormones exert some direct effect on water excretion, independent of their actions on electrolyte metabolism. The fact that DCA and other adrenal hormones inhibit the renal tubular reabsorption of water suggests the necessary mechanism. Such an idea would embrace current information concerning the opossum—a form which shows unorthodox effects of cortical hormones on electrolyte excretion but which demonstrates, nevertheless, the expected diuretic response to these hormones (217).

While the cortical hormone-induced diuresis of acute experiments in adrenalectomized animals seems to be unaffected by salt loads, the diabetes insipidus-like syndrome, induced by chronic administration of DCA in intact animals, although said to be "primarily a disturbance in water metabolism" (148), is at least to some degree dependent on salt intake. In the rat, the syndrome is demonstrable only slightly (205, 25) or not at all (171, 174) in the absence of some undetermined amount of dietary salt, but is readily seen when salt is given concomitantly with DCA (171, 25, 174). In the dog, the effect may not be entirely dependent on excess dietary salt, but it is so in its full-blown manifestations (148, 171). Other experiments demonstrate that in normal, adrenalectomized or hypophysectomized animals, the diuretic effects of DCA, chronically administered, were either dependent on or greatly increased by extra NaCl intake (41, 99). It is important, however, that all these examples of induced polyuria were associated with polydipsia and possibly secondary

to it. It has been suggested (148, 171, 174, 160, 3) that the action of DCA in this respect is primarily extra-renal in origin and results from a vicious cycle in which the steroid induces salt retention and thereby thirst, a process exaggerated by additional dietary salt. According to this concept polydipsia would be the immediate cause of the polyuria. Such ideas apparently would be consistent with the work of Rice and Richter (174).

To accept the hypothesis that the polyuria induced by chronic administration of DCA is primarily due to polydipsia does not, however, preclude the possibility that an additional contributory factor is a DCA-inhibited tubular reabsorption of water, although the latter factor may perhaps be overshadowed by a more massive polyuria induced by thirst. It is suggested, therefore, that the various cortical hormones enhance urine volume in two ways; *a) they augment thirst due to sodium retention.* In this effect DCA is the most active adrenal steroid because of its sodium-retaining action. Its diuretic effects consequently are best seen in chronic experiments, allowing time for a salt load to be built up, and are vastly enhanced by added dietary salt. In animals deprived of water, the effect may not be seen at all (158) unless extensive prior treatment with DCA (148, 276) has built up a water and salt load. *b) In addition, the cortical hormones inhibit tubular reabsorption of water.* This effect is essentially independent of salt load and is most easily seen under conditions of acute water overdosage, but can still be detected after water deprivation and dehydration (158).

If this hypothesis be granted, it would appear to permit correlation of most existing data on the subject—data which has hitherto seemed enigmatic (69). It also would permit intelligible recognition of the fact that DCA by a primary and dominant sodium-retaining action could, on the other hand, cause a reduced water excretion (148, 151, 193); such might be expected transiently in the absence of excess water when salt is retained before a polydipsia appears. There are other instances in which the action of DCA is reversed when the physiological conditions are changed, e.g., it depresses salt excretion in Addison's disease and enhances it in Cushing's Syndrome (225).

#### IV. RELATION BETWEEN ANTIDIURETIC HORMONE OF NEUROHYPOPHYSIS AND ADRENAL CORTICAL HORMONES

The theory that normal water balance is the consequence of a physiological antagonism between the diuretic action of cortical hormones and the antidiuretic action of posterior pituitary hormone was first stated (218, 217) and extensively elaborated (42, 216, 41, 20, 18) by Britton and collaborators. A great deal of additional work indicating the antagonistic action of cortical and posterior lobe hormones on water excretion (148, 131, 115, 4, 6, 272, 274, 268, 183), and as shown below on NaCl excretion, can be marshalled but the concept of a simple check-and-balance relationship should be approached with caution (210) until further evidence is available. It has not been shown, for instance, that secretions of the two glands are released temporally and quantitatively in a way suggesting an integrated physiological relationship. And while some of their actions are antagonistic, others are not, e.g., both DCA and Pitressin enhance potassium excretion at normal or low levels of potassium intake (194).



A possible new approach to the problem has been provided by the recent discovery that an antidiuretic substance, which behaves like posterior pituitary ADH, can be readily detected and measured in the blood serum of normal rats (13, 11) and other species (hamster and rabbit, 14; man, 132). The properties of this substance (13, 116, 11) suggest its identity with ADH: *a*) its antidiuretic action is effected by enhancing the tubular reabsorption of water although it transiently increases glomerular filtration; *b*) it is chloruretic; *c*) it is increased in dehydration and decreased by hydration; *d*) it is not detectable in the serum of hypophysectomized rats; *e*) it is, like Pitressin, quickly inactivated by neutralized thioglycolic acid. Its specificity is implied by the fact that isotonic gelatin solutions have no similar antidiuretic effects, while the antidiuretic actions of crude irritating protein solutions can be clearly distinguished from those of serum by the fact, among others, that they are not chloruretic. It will be interesting to explore the relation of this finding to that of the labile oxytocic substance found in human blood (43).

This antidiuretic substance was found to be increased after adrenalectomy (13), a result in harmony with the earlier report (144) that an antidiuretic substance appeared in the urine of cats after adrenalectomy. This presumably accounts in part for the increased tubular reabsorption of water and failure of water diuresis after adrenalectomy but on the basis of present evidence cannot do so completely. For instance, when large doses of water are force-fed to rats within 18 hours after adrenalectomy, deficiencies in diuresis are already evident but there is not yet appreciable accumulation of the antidiuretic substance. Secondly, when adrenalectomized rats are treated with saline or small doses of DCA the levels of antidiuretic substance are restored to normal but deficiencies in diuresis persist (6). There is another factor, however, which may be of greater importance than the elevated blood levels of antidiuretic materials in adrenal insufficiency. Properly devised tests have shown that within 18 hours after adrenalectomy there is a clearly enhanced sensitivity to the injection of antidiuretic substances (Pitressin) (13). An increased sensitivity to such materials would result in a depressed diuresis after water ingestion whether or not the amounts were above normal.

Lloyd's studies (132) attempting to correlate the blood levels of antidiuretic materials with corticoid excretion in a variety of clinical conditions, although incomplete as yet, offer a particularly promising approach to the problem. He finds low levels of unidentified antidiuretic substances in the serum of patients with diabetes insipidus, high levels in Addison's disease, and in general high urine volumes are associated with high corticoid excretion and vice-versa.

The early experiments of Martin and co-workers (144) led them to the conclusion—entirely consistent with their observations—that the presence of a urinary antidiuretic after adrenalectomy reflected a purposeful adaptation of neurohypophyseal activity to prevent dehydration. When it is considered, however, that forced fluids cannot be excreted at normal rates after adrenalectomy, it seems more probable that the organism is dealing with an unregulated aberration of normal function.

The recent report of Skahen and Green (209) that the chronic administration of DCA or salt leads to hypertension, polyuria and the excretion of an antidiuretic substance in the urine cannot at the present time be correlated with observations

noted above. It seems paradoxical that both an overdosage and deficiency of cortical steroid hormone should induce accumulation of antidiuretic substances in body fluids. Could it be, however, that the chronic administration of either DCA or salt would lead to a compensatory secretion of ADH to facilitate (teleologically speaking) the excretion of salt? There is abundant evidence of an antagonistic action of cortical and posterior pituitary hormones on NaCl secretion (131, 41, 42, 4, 152, 210, 194), just as there is on water excretion. The cortical substances, particularly DCA, cause NaCl retention and Pitressin enhances its excretion. Under conditions of rapid water diuresis a chloride-retaining action of cortical hormones is difficult to demonstrate, but the chloruretic action of Pitressin, considered either in terms of concentration or total amounts of chloride in the urine, has been seen by many above-mentioned workers. Questions both of fact and interpretation have been raised, however, in regard to such results (85, 161, 86). The demonstration by Winter, Ingram and Gross (274, 268) that the depletion of plasma sodium which follows adrenalectomy in cats is dependent upon a functional posterior pituitary is of great interest and significance in this connection. The implication of these various results is that the posterior pituitary is fully as important as cortical hormones in regulating NaCl metabolism and that normal salt and water excretion may result from a balanced interaction of the two glands. It is questionable whether this theory will explain the increased tubular reabsorption of sodium that occurs in adrenalectomized dogs when high plasma levels of sodium are maintained (183).

No satisfactory information is available as to the cause of the accumulation of antidiuretic substances in the blood of adrenalectomized animals. It could be due to an increased posterior pituitary activity, although Gersh and Grollman (80) failed to find cytological evidence of such an increase, or to an arrested rate of inactivation of ADH. The liver is known to be involved in the inactivation of ADH (103, 52) and liver functions in other respects are altered by adrenalectomy (185, 248) and cortical hormones (221). While the possibility of causal relations is suggested, evidence is lacking. Current theory has it that ADH is released by the stimulus of a rising plasma osmotic pressure (29, 27, 259). It is highly unlikely that such factors are operative after adrenalectomy, particularly after the ingestion of excess water where most important osmotically active substances are reduced in concentration. Even if it be granted that osmotic stimuli are not present to activate the release of ADH, other stimuli might conceivably do so. It is known, for instance, that neurogenic as well as osmotic stimuli may excite the neurohypophysis (17, 156, 98).

The hypophysectomized animal is an experimental form of interest from the standpoint of possible antagonism between the adrenal cortex and posterior pituitary. Here one gland is extirpated and the other largely inactivated from lack of its trophic hormone. Unfortunately the situation is complicated by the fact that non-adrenotrophic anterior pituitary hormones are also lacking. Whatever may be true of the daily water exchange and the phenomenon of diabetes insipidus after total hypophysectomy, as discussed below, one fact is clear: the diuretic response to forced water is sluggish after hypophysectomy as it is after adrenalectomy (263, 32, 119, 165, 157); the rate of extra-renal water loss—itself low in the absence of the anterior pituitary—exceeds the rate of renal excretion (119); and the deficiency responds

markedly to cortical hormone therapy in the rat (119, 157) if not the dog (165). The renal abnormalities of hypophysectomized dogs, in other respects, are not repaired by cortical hormones (267). In addition, there is a marked susceptibility of hypophysectomized rats (77) and frogs (197) to water intoxication and an abnormal response to the fluid shifts induced by the injection of intraperitoneal glucose (118) which resemble to some degree conditions seen in adrenal insufficiency. This led to the hypothesis that the failure of water diuresis was due to identical causes in both adrenalectomy and hypophysectomy (119). Recent work requires that this hypothesis be revised. As noted earlier, the adrenalectomized animal has a higher than normal amount of antidiuretic substance in the blood; the hypophysectomized animal, on the other hand, has none (11). The two specimens are therefore fundamentally different as far as the factors acting on water metabolism are concerned. These facts permit understanding of the hitherto anomalous observation (119, 157) that the diuretic response to water is restored by cortical hormones more easily in hypophysectomized than in adrenalectomized animals. In the latter, any injected material has to counteract a large amount of circulating antidiuretic substance; in the former it can act unantagonized. In the absence of known agents inhibiting water excretion it is surprising that the hypophysectomized animal cannot excrete large volumes of water readily. One can only state in explanation the general rule that rapid water excretion is apparently never possible in the absence of cortical hormones. These facts do not fit comfortably into the simple hypothesis that the rate of water excretion is determined only by the relative amounts of cortical and posterior pituitary hormone present in the blood. If both are absent rapid diuresis is still impossible.

Wesson, Anslow and Smith (265a) have offered an hypothesis relating the action of ADH to sodium and water excretion and to the regulation of extracellular volume. They did not attempt to fit the action of cortical hormones into their scheme.

#### V. RELATION OF ADRENAL CORTEX, ANTERIOR PITUITARY AND POSTERIOR PITUITARY IN DIABETES INSIPIDUS

Another approach to the relation of cortical and pituitary hormones in water metabolism is that provided by the extensive studies on experimental diabetes insipidus. It is now generally accepted that full-fledged diabetes insipidus results when a neurohypophyseal mechanism is damaged or inactivated and the anterior pituitary functional (262, 79, 179, 58). When the whole pituitary is removed a transitory diabetes insipidus develops which within a few days may subside to negligible proportions. Disturbances in water metabolism persist, however, as indicated by a low ratio of water intake to urine output (199), by a persisting inability to excrete a concentrated urine (162, 198), and by a diminished diuretic response to water ingestion in acute experiments (Sec. IV).

Of particular interest for this discussion is the nature of the essential rôle of the anterior pituitary in the maintenance of diabetes insipidus. No specific anterior lobe diuretic hormone has been found, and most workers feel that the effect is mediated by the indirect action of one or more of the known anterior lobe hormones (177).

The theory that ACTH, acting through the adrenal cortex, is the diuretic agent of the anterior pituitary was first stated by Britton *et al.* (218, 217). Other workers (198, 199, 272, 105) have not accepted the idea in toto. The implication of much of the evidence cited in foregoing sections of this review is, however, that the adrenal cortex must be involved to some degree in the maintenance of diabetes insipidus. The important points of argument can be briefly stated as follows:

1) The cortical hormones are natural diuretic agents and are essential to the full action of other natural diuretics such as thyroxin (198, 70) and epinephrine (73). Since anterior pituitary removal largely inactivates the adrenal cortices, the absence of full-fledged diabetes insipidus after hypophysectomy is to be expected. 2) The removal of the adrenals diminishes the diabetes insipidus established by appropriate hypothalamic lesions (115) and overdosage with DCA accentuates the water exchange characteristic of diabetes insipidus (99). 3) Cortical hormones, particularly DCA (198, 152, 205, 104, 41), will partially restore a state of diabetes insipidus in hypophysectomized animals but this action is not as quick or complete as would be expected if this were the sole influence involved (198). 4) In intact dogs, chronic administration of DCA can induce a diabetes insipidus-like syndrome (Sec. II).

The evidence previously cited of adrenal cortical-posterior pituitary antagonism leads logically to the concept that total hypophysectomy, by removing or functionally inactivating these two glands, attenuates diabetes insipidus in a manner analagous to that in which hypophysectomy attenuates diabetes mellitus. To admit the importance of these adrenal cortical factors does not, however, permit dismissal of impressive evidence that other factors are involved in diabetes insipidus. The thyroid, which is more completely inactivated than the adrenals by hypophysectomy, is an important agent in water metabolism and its rôle in diabetes insipidus has been amply demonstrated (58, 105, 121, 23, 33).

The growth hormone of the pituitary affects water metabolism as an essential feature of its growth-promoting action and in addition has profound effects on renal function (267a); it thus is probably involved in this problem. Since the animal without a functional neurohypophysis cannot concentrate urine, any pituitary hormone which stimulates appetite or metabolism in such a way as to require the excretion of additional metabolites must cause added water excretion. The enhancement of diabetes insipidus when additional salts (232, 273, 233, 275) or urea (273, 271) have to be excreted has been frequently observed.

There are, therefore, several anterior pituitary hormones which tend to produce polyuria. When they are unchecked by ADH, diabetes insipidus is probably the resulting sum-total of their activities.

#### VI. EXTRA-RENAL ASPECTS OF ADRENAL CORTICAL REGULATION OF WATER METABOLISM

The previous sections have been concerned largely with factors affecting the excretion of water and electrolytes. Such factors lead obviously to changes in internal fluid distribution. Some of the internal changes which follow adrenalectomy are secondary to altered renal functions and some are not. The distinction is oftentimes difficult to make. Here again the problem involved is that of the intrinsic or self-

contained antagonistic properties of steroids such as DCA; they cause sodium retention and water retention, while at the same time affecting kidney tubules in such a way as to stimulate water excretion.

Earlier reviews (241, 95, 122) contain more complete accounts of the older work on this subject than will be attempted here.

*Internal Fluid and Electrolyte Distribution; Water Intoxication; Intestinal Absorption.* An appearance of dehydration is a commonly described symptom of adrenal insufficiency (224). A careful study of the total body water in the adrenalectomized rat, however, revealed no significant reduction in the percentage of water determined on a fat-free basis (87). There is a reduction in blood volume (93, 281, 239, 96) and of extracellular fluids in general (93, 91). While it has been suggested that the loss of blood volume is probably due to loss of fluid through the kidneys (93, 91), this explanation is open to question as it has been estimated that the water and NaCl (59) loss from the extracellular fluid is greater than can be accounted for on the basis of renal excretion (235, 241, 90). A shift of water from extra- to intracellular sites would be expected if this were true and has been found (90, 46). The latter is reflected by an increased water content of all tissues studied: muscle (261, 214, 212, 45, 150), skin (94), liver (214, 94), red blood cells (149, 102).

Overdosage phenomena correlate well with the above findings. Many observers have reported an increased blood volume following DCA administration in intact animals (3, 236, 170, 37, 36), while others have observed that various adrenal steroids prevent the decline in blood volume after adrenalectomy (236, 19, 129, 127). In the case of DCA administration this is associated with sodium retention and potassium loss, and after prolonged treatment with the substitution of sodium for intracellular potassium (55). It has been reported (65) recently that DCA given to normal dogs caused an increase in the extracellular space with no change in total body water. This means that there was a decrease in the intracellular water. In contrast, dogs treated with cortical extract showed no significant change in the extracellular volume but the total body water, and hence intracellular water, increased markedly.

The effects of DCA on both electrolyte and water retention are apparently antagonized to some extent by other cortical secretions (Sec. I), a fact which serves as the basis for the observation that edema is easily produced by DCA therapy in Addisonian (56) but not in normal patients (224, 249). Similarly, an excess of natural adrenal secretions presumably accounts for the fact that in Cushing's Syndrome, DCA enhances the rate of excretion of intravenous NaCl solution whereas it facilitates its retention and consequent edema formation in Addisonians (225).

A definite example of the extra-renal origin of some phenomena of this type is seen in water intoxication. Adrenalectomized animals show a sensitivity to excess water not wholly due to deficient water excretion (242, 100, 68). That point was unmistakably made by the demonstration that after nephrectomy DCA greatly increased the resistance to high water loads (12). An analogous resistance to water intoxication, apparently not due entirely to increased diuresis, was seen in animals adapted to the receipt of water by stomach tube (130, 247). Such adaptive phenomena were not manifest after adrenalectomy (130). The protective action of cortical hormones against excess water in normal animals (66), however, is due largely to renal factors (12). The nature of the extra-renal actions of the cortical hormones in water

intoxication is not clear but much basic information has been reported (181, 238, 242, 68, 100, 211, 12) and summarized (69). Briefly, marked differences have been reported between normal and adrenalectomized animals given excess water in the maintenance of body temperature, hematocrit values, plasma electrolyte levels, intestinal absorption, etc. These differences are only partially explicable by renal activity and in general suggest an abnormal internal distribution of ingested fluid after adrenalectomy. Convincing exposition of the nature of that difference has not been made and current opinions, even as to the site of retained fluid, are not in complete agreement (68, 211, 100, 69).

Water intoxication is generally thought to be due to cellular hydration resulting from a reduction by dilution or otherwise in extracellular electrolyte. It can be prevented by NaCl administration (184, 84, 67). It might be expected, therefore, that the cortical hormones would prevent water intoxication by maintaining high plasma electrolyte levels due to their usual salt-retaining actions. It is a curious fact that they do not exert their effect, whatever it may be, by this means (238, 66, 12) either in normal or adrenalectomized animals. On the other hand, thyroxin, which also prevents water intoxication, does probably act in part by causing salt retention (70).

Another unexpected fact is that lethal water intoxication is not associated with evidence of circulatory collapse in adrenalectomized animals (238, 68) as is the case with most other stresses of similar severity. Circulatory abnormality of a more subtle sort, however, is a possible cause of the recent finding that there is a sharp fall in renal glomerular filtration when adrenal deficient animals are forced into water intoxication (14).

One sharply defined extra-renal factor is a deficiency in the intestinal absorption of sodium salts and water after adrenalectomy (34, 68, 47, 229) and hypophysectomy (119). This could be a major cause of the sluggish water diuresis but actually is probably of minor importance; as indicated in earlier sections, fluid given by parenteral routes is excreted as poorly as that which has to go through the gut.

*Permeability.* Viale and Bruno (261) observed a decrease in the plasma volume of adrenalectomized dogs and suggested increased vascular permeability as a possible explanation. Other early work implied changes in capillary permeability in adrenal insufficiency (239). The failure of serum transfusions to protect adrenalectomized animals against circulatory collapse, and the appearance of edema following such transfusions (236), indicated increased capillary permeability. Cope *et al.* (40) observed an increased protein content of lymph collected from the cervical trunk of dogs after adrenalectomy—a result probably indicating changes in capillary permeability. Hyman and Chambers (110) found that various cortical steroids helped prevent edema in the perfused legs of frogs. It has been reported (269) and questioned (167) that muscle tissue of adrenalectomized rats and of frogs (5) imbibes water faster in hypotonic solutions and loses water faster in hypertonic solutions than do muscles taken from normal animals. These findings may have been the result of equilibrium phenomena caused by differences in initial electrolyte content of the tissues studied rather than manifestations of permeability changes *per se*. A large volume of experimental evidence (145, 61, 57, 241, 95) is concerned with changes in permeability to dye substances after adrenalectomy.

While much has been said or implied concerning the effects of adrenal cortical

hormones on permeability, knowledge of the extent and nature of such phenomena is far from satisfactory. Most convincing evidence concerns capillary permeability to proteins. Whether these effects are all due to permeability changes *per se* or to changes in circulatory dynamics is open to question (30). The problem of possible changes in cellular permeability to electrolyte and other ions remains unanswered. While it is clear that cortical hormones bring about changes in ionic distribution between cells and extracellular fluids (Sec. I), these seem to be the direct result of alterations in the electrolyte content of extracellular fluids, a consequence of renal activity, rather than an expression of hormone action on cellular permeability.

#### VII. COMPARATIVE ACTIONS OF DIFFERENT STEROIDS ON WATER METABOLISM; REPLACEMENT THERAPY

Although DCA has long been recognized as the cortical steroid that acts most effectively on the kidney to inhibit salt excretion and increase potassium excretion and has been known as the prototype of the adrenal 'salt and water' hormones, it is not the most effective in stimulating diuresis in acute tests or in preventing water intoxication. One mg. of 17-hydroxy-11-dehydrocorticosterone (compound E) has a potency about equal to 3 mg. of DCA in maintaining diuresis and preventing toxic symptoms in the adrenalectomized rat given large doses of water (54). Under similar conditions whole adrenal cortical extract is clearly superior to DCA when used in doses standardized to equivalence in life and growth maintenance tests (54). Cortical extract is much more effective than DCA in maintaining the diuretic response to ingested water in hypophysectomized rats (119, 157). It has also been found that while both cortical extract and DCA will protect normal rats from water intoxication the former is apparently more effective (66). In mildly dehydrated animals, the diuretic action of cortical extract has been seen under conditions where DCA is ineffective (158). While further data on the point are needed, it appears now, on the other hand, that DCA is more active than other cortical substances in inducing high rates of water excretion after chronic administration in animals allowed free access to water. Under these conditions a polydipsia is induced that leads to a diabetes insipidus-like syndrome, likely due in part to salt retention (Secs. II, III).

Despite such quantitative differences, all cortical substances which have been tried (DCA, 17-hydroxy-11-dehydrocorticosterone, the amorphous fraction and whole adrenal extract) can stimulate diuresis (54). Progesterone is the only substance exhibiting marked corticoid activity reported to be without effect on diuresis when given acutely in water tolerance tests (75). Re-study of this point is appropriate since it has been shown (206) that, after chronic administration, progesterone enhances water exchange in both normal and hypophysectomized rats and increases the rate of elimination of water given by stomach tube. In the hypophysectomized animals it also increased the resistance to water intoxication (206).

The estrogens and androgens share with many other steroids the property of reducing the excretion of sodium and of causing water retention (252). Like DCA and progesterone they also cause reduction of serum and muscle potassium (146). The estrogens differ from progesterone in that they are toxic to adrenalectomized animals (195, 26, 72). Both estrogen and testosterone differ from progesterone in

that they do not provoke diuresis under any conditions yet observed (75, 205). There is as yet no basis on which all of these facts can be correlated satisfactorily, but probably all steroid hormones affect salt and water metabolism in some way.

One of the most curious aspects of the whole problem is the difficulty of repairing the deficiencies in water diuresis of adrenalectomized animals, and Addisonian patients. Replacement therapies are relatively effective if used in the early stages after adrenalectomy (54, 100), but not if 3 or more days are allowed to elapse after operation. Even when animals are treated with salt, DCA or cortical extract beginning immediately after adrenal removal it has generally not been possible to restore completely the diuretic response to water (100, 142, 68, 82). The same problem has appeared in treated Addisonian patients (128, 69, 280). Large doses of cortical substances are of some effect (68, 100) and it has recently been found (14) that if adrenalectomized rats are treated with 5 mg. DCA per day for 5 days a normal excretion of ingested water is obtained. The dog apparently responds better than other forms (242, 123). The difficulty of replacement is likely related to that of other persisting abnormalities of renal function in Addisonian patients on otherwise adequate therapy (245, 264). Despite the difficulty of restoring diuresis, cortical hormones are, however, effective in preventing symptoms of water intoxication in adrenalectomized animals by their extra-renal action (Sec. VI).

#### VIII. RELATION OF ADRENAL CORTEX AND MEDULLA IN WATER METABOLISM

In most experiments on water metabolism done with adrenalectomized animals, the adrenal medulla as well as the cortices have been removed and this fact in itself makes inquiry into the possible effects of medullectomy imperative. The recent findings of Verney and associates (260) that epinephrine inhibits the emotional release of ADH adds interest to the problem. (Is it possible that the Verney phenomenon could be due to the fact that epinephrine injections, as is well known, induce the release of ACTH and that the resulting secretion of adrenal cortical hormones antagonizes ADH?)

Some time ago, it was shown that rats in which one adrenal was removed and the other demedullated excreted excess water less rapidly than normal animals. While this deficiency could have been due to the lack of medullary tissue, the fact that it was fully corrected by the use of adrenal cortical extract led to the interpretation that it was rather the result of subnormal functional capacity of the remnant of cortical tissue (53). Obviously more conclusive experiments were needed. The adrenal medulla is activated by water overdosage as indicated by a hyperglycemia which fails to occur after medullectomy (68).

Stein and Wertheimer (230) made the first systematic study of the problem with the finding that medullectomized rats had a deficient diuretic response to water and increased susceptibility to water intoxication. These deficiencies could be repaired, not only in medullectomized but also in totally adrenalectomized rats, by epinephrine injections. Epinephrine increased chloride excretion, particularly in medullectomized rats. Somewhat variable alterations appeared in the daily water exchange of animals from which the adrenal medullae were removed.

While Stein and Wertheimer's interpretations of these findings were conservative,



it is obvious that many of the results previously attributed to cortical deficiency might in fact have been due to medullary deficiency.

Reexamination of this question was made in two laboratories. It was found in rats given variable doses of water, that epinephrine would enhance diuresis, in some cases to a remarkable extent, in normal (73) medullectomized (73) and adrenalectomized (73, 100) rats, and in all groups would enhance chloride excretion (73). The medullary hormone was less effective in preventing water intoxication than expected, perhaps because of its deleterious stimulation of salt excretion.

That the results were of great physiological significance was questioned (73) largely on the basis of the fact that it was not confirmed that demedullated animals responded to forced water in any way different from normal ones, i.e. no deficiency phenomena were observed. Furthermore, these effects of epinephrine injections were seen only when dosage was high enough to produce obvious overdosage symptoms. Amounts of epinephrine which produced no overt overdosage symptoms failed to affect diuresis (73). Scattered reports have been obtained which indicate that epinephrine is of no avail in restoring the diuretic response to water in human cases of adrenal insufficiency (69).

While it is the authors' opinion that the adrenal medulla is not involved in any important respect in the alteration of water metabolism associated with adrenalectomy, there are admittedly unsolved aspects of the problem of great interest. For instance, epinephrine administration may induce either diuresis or antidiuresis and the pharmacology of these different actions is vague (175, 176, 256, 260, 190, 31, 277). It is one of the few non-steroid agents which exerts a clearly diuretic influence in adrenalectomized animals and this action is apparently a specific one; at least other related drugs will not duplicate epinephrine action (230).

#### IX. RELATION OF ADRENAL CORTEX AND THYROID IN WATER METABOLISM

It has long been recognized that the thyroid influences water metabolism (163, 164), and specifically that thyroxin stimulates water diuresis (164, 70). Since the adrenal cortical hormones stimulate diuresis and thyroxin increases adrenal cortical activity (246, 137) it is obviously possible that the thyroid action might be mediated through the adrenals. Evidence on that point is not conclusive but as shown below it is clear that while the thyroid influence is not exerted entirely through the adrenals, it cannot manifest itself in the absence of cortical hormones.

When animals treated with thyroxin are given water by stomach tube they excrete it rapidly and if a high water dosage is used, a marked resistance to water intoxication is seen (70). This is partly due to the fact that the hyperthyroid animal can excrete large volumes of water of very low chloride, and presumably sodium content (169, 70), an effect which in water intoxication would have the same protective action as that of the administration of salt. This salt conservation is not due to adrenal influence since under similar conditions adrenal cortical hormones do not decrease the chloride excretion (66). Nevertheless, the accelerated diuresis is largely abolished by adrenalectomy and is therefore dependent in some way on adrenal function (70). The effects of hyperthyroidism in man are apparently the opposite of those in animals and lead to decreased rather than increased water tolerance (128). Such

results could be readily explained if hyperthyroidism led to adrenal exhaustion in one species but not another; the question would be an interesting one to explore. It has, in fact, been reported that a reduction in adrenal size may characterize human hyperthyroidism in contrast to the usual hypertrophy in animals (126).

Thyroid hormones affect various renal functions (104, 51) but the possible relation of these actions to the cortical hormones and water metabolism is not clear.

Curiously enough the response to either small or large doses of water is completely normal in hypothyroid rats whether that hypothyroid condition is induced by surgery (101) or thiouracil (70).

#### X. RELATION OF ADRENAL CORTEX TO ALTERATIONS IN WATER METABOLISM SEEN IN ADAPTATIONAL SYNDROME

When animals are subjected to stimuli which invoke intensive adaptive responses, such as exposure to cold, traumatic shock, drugs, toxins etc., the initial or 'shock phase' of the syndrome is characterized by the retention of administered fluid (107, 120, 108, 6, 22). This condition lasts for approximately 24 hours and is followed by a reverse effect which appears during or just after the 'counter-shock phase' (6, 22, 24). Although careful analyses have not been made of the initial phase characterized by an antidiuretic response to water it is probably of complicated origin; the general shock-like symptoms, hypotension, hemoconcentration, possible increased activity of the neurohypophysis, and possible transient adrenal cortical deficiency due to the rapid utilization of the hormone all may be contributing factors. The latter factor is mentioned because at least in some instances diuresis can be restored (108, 107, 69), and symptoms improved (207) by cortical hormones. During the initial antidiuretic reaction elicited by a stress-producing stimulus the application of a second stress fails to cause further water retention but rather provokes an increased urine output (120, 108, 107).

Within two days after a damaging stimulus has been applied, during what Selye (201, 202) would term the 'stage of resistance,' there is a definite acceleration of the diuretic response to administered water (6, 22, 24). Considering that adrenal enlargement (204, 112) and increased corticoid excretion (258, 265, 166) follow exposure to a wide variety of stress-producing agents (204, 112), it is possible that the diuretic reaction is due to hyperactivity of the adrenal cortex. Further evidence as to the rôle of the adrenal cortex in provoking the diuretic reaction is found in the observation that the response is abolished by adrenalectomy (208, 200, 204) or hypophysectomy (200, 204), and is very closely duplicated in normal animals by adrenal cortical extract (6, 16).

#### XI. ACTION OF CORTICAL HORMONES IN EPILEPTIC AND OTHER CONVULSIONS RELATED TO TISSUE HYDRATION

McQuarrie and Peeler (154) introduced and others have used (64, 106) a test for idiopathic epilepsy based on a susceptibility to seizures following a low salt diet and acute hydration. In electro-shock and drug-induced convulsions, increase in cellular hydration lowered the threshold to psychomotor seizures and changes in extracellular fluid volume, when unaccompanied by changes in electrolyte concentra-

tion, had no effect on such thresholds (244). Later work suggested that the extracellular sodium and chloride concentrations were more important than several other factors studied (243). The implication that the susceptibility to convulsions is related to such phenomena suggests a possible therapeutic value of a hormone such as DCA which causes sodium retention and helps maintain extracellular fluid volume.

It has been reported (153) that DCA will antagonize the seizure-provoking effects of Pitressin hydration in the epileptic subject and possibly, although there is lack of agreement on the subject (1), decrease the tendency for spontaneous occurrence of grand mal seizures. It likewise increased the threshold to electroshock seizures in the rat (279, 226), an effect which was surprisingly antagonized by adrenal cortical extract or ACTH (279). In the cat, it afforded protection against convulsions induced by cocaine but not electricity (1), and in the rat prevented both convulsions and pulmonary edema after metrazol treatment (203). Such actions resembled those of DCA in preventing water intoxication symptoms, among which are violent convulsions. This relation is further indicated by the fact that the alterations of the electroencephalogram are similar in water intoxication and epilepsy and certain phases of the former at least can be relieved by DCA (78). While the beneficial effects of DCA in epilepsy have been attributed to NaCl retention (153), it is not clear that the same explanation will suffice in water intoxication (12).

Although no comprehensive statements concerning the relationship between these various convulsive states and adrenal cortical function in electrolyte and water metabolism can be made as yet, the possibilities suggested are intriguing. This is particularly true in view of other accumulating evidence, not obviously germane to this review, that cortical hormones have important functions in regulating the electrical activity of the brain (60).

## XII. WATER DIURESIS TESTS OF ADRENAL CORTICAL FUNCTION

Many of the deficiencies which occur in adrenal insufficiency have been used as bases for the bioassay of adrenal cortical preparations.

Despite its mechanical simplicity no thorough study has been made of the possible usefulness of the deficient diuretic response to water and its repair, although one report (159) attests its value. It is apparent from published work that dose-response relationships of some sort could be obtained (54). It would be one of the most generalized tests for corticoid activity since most corticoids stimulate diuresis (Sec. VII). One obvious difficulty would lie in the poor responsiveness (Sec. VII) of animals that have been operated on 3 or more days previously. This might be eliminated, however, by basing tests on the enhanced diuresis which follows cortical hormone administration in intact animals.

Wide use has been made, however, of the deficiency in diuresis as a means of testing adrenal function. More or less apart from the concomitant animal work, the clinical findings of Rowntree and Snell (185) and Maranon *et al.* (139) preceded the widely used Robinson-Power-Kepler (182) 'water test' or 'Kepler test' for Addison's disease. The first part of this test simply applies the fact that in the absence of adequate amounts of cortical hormone, ingested water is not excreted at a normal rate. If it is excreted at a normal rate, Addison's disease can be ruled out in most instances. If excretion is deficient, factors other than Addison's disease must, of course, be

eliminated. More recent studies concern the physiological basis, application and reliability of the test (172, 128, 69, 280, 219). As is true in animals (Sec. VII), a normal response to the test is not restored by ordinary replacement therapy. It is apparent from animal work that the accuracy of the procedure could be considerably enhanced if doses of water larger than those recommended (2% of the body weight) could be forced on the human subject (69). Unfortunately, most subjects cannot tolerate larger doses.

### XIII. CONCLUSIONS

The following is offered as the authors' tentative interpretations of the data reviewed. Like most hypotheses in a rapidly developing field, they will certainly have to be revised as further information accumulates.

The hormones of the adrenal cortex have an important rôle in water metabolism, which is mediated, broadly speaking, in three different ways. *First*, there is a direct action on renal mechanisms, essentially independent of electrolyte metabolism, the effect of which is to stimulate the rate and extent of water excretion. *Secondly*, most cortical hormones cause sodium retention by the kidney, the osmotic consequence of which is a retention of water. Whether increased or decreased diuresis results from the interaction of these two factors depends upon the physiological conditions prevailing at any particular time. In the excretion of sodium chloride and water, the effects of cortical hormones are the opposite of those of the posterior lobe of the pituitary gland. More data is needed to establish the hypothesis that the adrenals and neurohypophysis operate as a coordinated antagonistic system but to a certain extent they probably do so. Shannon states "... both hormones are involved in the control of sodium balance. However, the action of the two does not appear to be integrated in the ordinary sense of the word" (210). The problem invites further attention. *Thirdly*, there are vaguely-defined extrarenal means by which the cortical hormones affect internal fluid distribution.

*Diuretic Action of Cortical Hormones.* The normal diuretic response to water, when water is given by any means or with any solute, depends upon the action of cortical hormones. Diuretic agents or influences thus far studied can act to their full capacity only in the presence of cortical hormones. Deficiencies in diuresis are apparent when gross symptoms of adrenal insufficiency are absent. Adrenalectomized animals can, of course, excrete water, but not at anything approaching a maximal normal rate. The water excretion of intact animals, even when dehydrated, is increased by the administration of cortical hormones. It is curiously difficult to obtain an effective replacement therapy and restore normal rates of water excretion either in animal or human subjects with adrenal insufficiency.

Actions of the cortical hormones on water excretion can be demonstrated which are not in any apparent essential way related to electrolyte stores or electrolyte excretion. All cortical substances studied have a diuretic influence, despite their variable effects on salt excretion, but compound E is a more effective diuretic than DCA. This means that substances most effective in inducing diuresis are least effective in causing salt retention, and this fact again points to the divorcement of the two actions.

Two actions of the cortical hormones are responsible for their direct effects on

water diuresis. *First*, they are necessary for the maintenance of a normal renal glomerular filtration rate. This is associated with the maintenance of a normal renal plasma flow. The factor of glomerular filtration, however, is of minor importance, since filtration can be restored to normal in situations where deficiencies in water diuresis persist. *Secondly*, the cortical hormones inhibit the renal tubular reabsorption of water and this is the *sine qua non* of their diuretic action. Tubular reabsorption exceeds normal in adrenal insufficiency and is less than normal when intact animals are overdosed with cortical hormone.

*Relation of Adrenal Cortex and Posterior Pituitary.* The probable cause of the increased tubular reabsorption of water in adrenal insufficiency is an accumulation of, or an increased responsiveness to, circulating antidiuretic substances. Increased amounts of antidiuretic material have been seen in the urine of adrenalectomized cats and in the blood serum of adrenalectomized rats and Addisonian patients. Considerable evidence points to their pituitary origin. In any case, when the diuretic cortical hormones are absent, there is an increased sensitivity to posterior pituitary ADH. A subnormal output of ADH, theoretically at least, could bring about, therefore, antidiuresis during adrenal insufficiency because it would be acting in the absence of a normal antagonist.

An important distinction should be drawn, however, between the analagous antidiuretic rôle of ADH and the diuretic rôle of cortical hormones: ADH is secreted at a variable rate and is sensitively responsive to varying states of hydration. At present there is no evidence to show that mild fluctuations in water load alter the rate of cortical hormone secretion, although such possibilities have not been excluded. Within fairly wide limits, an increased output of ADH may antagonize the effects of cortical hormones and maintain water balance at a more or less normal urine volume. ADH administration, however, is much less effective in controlling a diabetes insipidus-like condition induced by DCA than it is in ordinary diabetes insipidus. This is a counterpart of the increased effectiveness of ADH after adrenalectomy. Presumably, then, when the cortical hormone level is high enough, water balance can only be attained by increased water intake. According to this hypothesis, the cortical hormones would be essential for diuresis, but the finer regulation of water exchange would depend on factors controlling thirst mechanisms and ADH secretion. When both the corticoids and ADH are reduced in body fluids, as in hypophysectomy, rapid diuresis is impossible although readily restored by cortical extract. This demonstrates again the essential rôle of the cortical hormones for a high rate of water excretion, whatever may be the nature of their interacting function with ADH.

In diabetes insipidus, the action of cortical hormones, uninhibited by ADH, is almost certainly contributory to the observed polyuria. Anterior pituitary factors, other than the ACTH-adrenal cortical system, however, seem to have important influences as well. The essential rôle of the anterior pituitary in maintaining a state of diabetes insipidus when the neurohypophysis is removed or inactivated is best explained as being due to multiple influences the sum total of which achieves polyuria.

*Water-Retaining Action of Cortical Hormones.* Most cortical hormones, and particularly DCA, cause retention of sodium salts. This leads to an osmotic retention of water, increased extracellular fluid volume and possibly frank edema. In the early

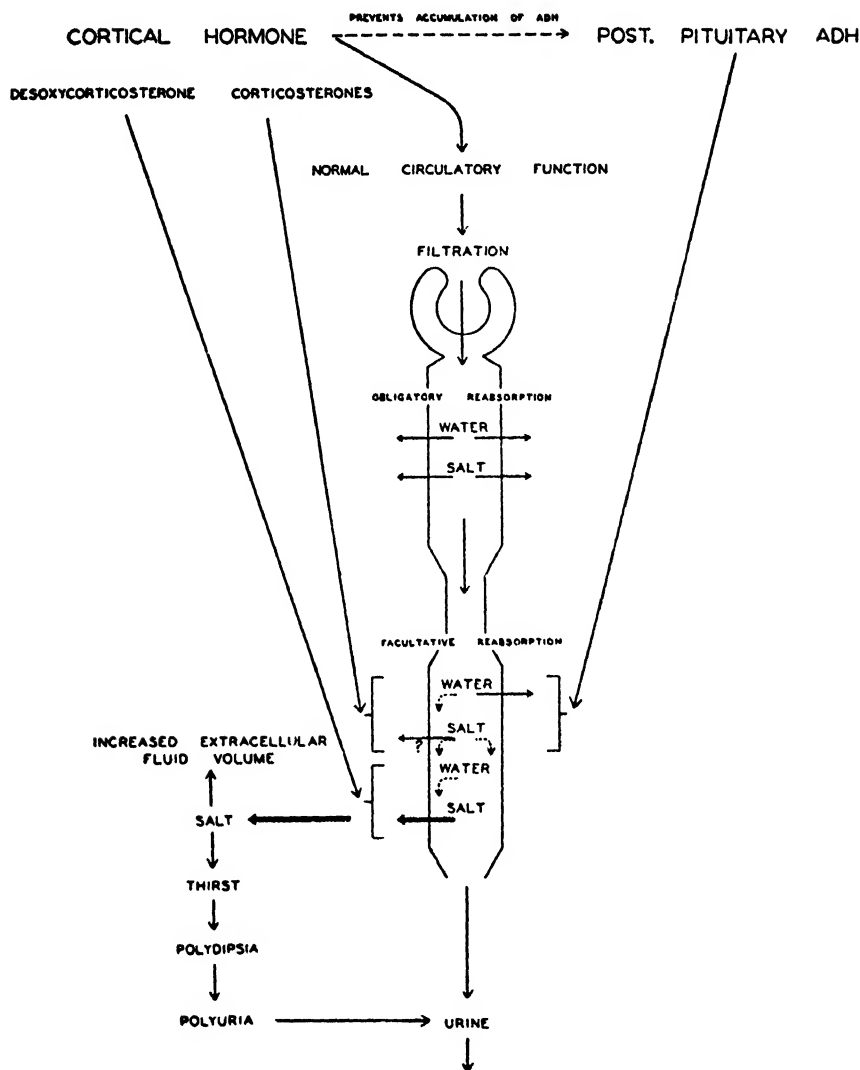


Fig. 1. INDICATING POSSIBLE PATTERN of interaction of adrenal cortical and posterior pituitary hormones in salt and water metabolism. According to this scheme ADH stimulates the tubular reabsorption of water and inhibits (broken arrow) the reabsorption of sodium and chloride. The cortical hormones a) prevent the accumulation of antidiuretic material, presumably ADH, in body fluids by mechanisms as yet unknown; b) maintain renal plasma flow and glomerular filtration; c) inhibit the tubular reabsorption of water; d) in some instances and conditions inhibit and in some stimulate the reabsorption of NaCl; e) when sodium is retained it causes expansion of the extracellular fluid volume, thirst, and eventually, acting together with other factors, augments the water exchange.

stages after cortical hormone administration, if excess water is not forced on the subject, this water-retaining effect may override the diuretic effect of inhibited tubular reabsorption and result in reduced urine volume. If treatment is continued, however, the retention of salt is associated with the development of thirst which

leads to a diabetes insipidus-like state—a state probably due to a combination of increased thirst and inhibited renal tubular reabsorption of water. When the experimental circumstances are such that these factors can all operate simultaneously, a water retention, i.e., an expanded extracellular fluid volume, and an increased water diuresis can occur simultaneously. Thus, there is no essential inconsistency in the statement that cortical hormones promote both water retention and water loss (fig. 1).

*Extrarenal Factors.* The extrarenal actions of cortical hormones on such phenomena are too poorly understood to be fitted into this scheme as yet. Their absence is associated with evidence of an altered internal distribution of fluid and a relatively unimportant reduction in the absorption of water from the intestine. Whatever may be their nature, these extrarenal effects are of such magnitude as to make the difference between survival or death when water is given nephrectomized animals. They presumably account for the marked sensitivity to water intoxication in adrenal deficient animals, a sensitivity that results in the appearance of toxic symptoms at relatively light water loads. Further study of the relation of these phenomena to permeability, the behavior of body electrolytes, circulatory function etc., is needed.

*Significance of Desoxycorticosterone.* Because of its availability by synthesis much of the work on this and related adrenal subjects has been based on the use of desoxycorticosterone. This substance, however, has been found in adrenal tissue in only one laboratory (173) and then in minute amounts. The question can be raised, but not answered at present, as to whether it is an adrenal hormone of physiological importance. Whether it is or not affects the conclusions of this paper only as regards the water-retaining aspects of the subject. Salt and water retention, and their sequelae, are brought about more effectively by DCA than other crystalline adrenal hormones, and exaggerated importance might, therefore, be attributed to them by studies in which this steroid was used. Unidentified sodium-retaining materials are present in cortical extracts (95, p. 93) but they are not identical with desoxycorticosterone.

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# MOTION SICKNESS

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MOTION SICKNESS is a specific disorder which is evoked in susceptible persons and animals when they are subjected to movements which have certain characteristics. Irwin in 1881 (115) appears to have been the first to use the expression. He suggested that seasickness might more correctly be termed motion sickness "for not only does it occur on lakes and even on rivers, but as is well known, a sickness identical in kind may be induced by various other motions than that of turbulent water, . . ." He thus introduced a convenient and accurate general term, but it did not gain wide use until the beginning of World War II when the late Sir Frederick Banting grouped under the term motion sickness "a variety of conditions akin to seasickness and due to frequently repeated oscillatory movements of the body" (153). While there is some evidence that visual reception of motion may produce a similar sickness in individuals of special susceptibility when their bodies and heads (but probably not their eyes) are motionless (see below), it is questionable whether this response should be classified under the same term, for it is etiologically distinct.

The literature on motion sickness which appeared before World War II contains amazingly little factual information concerning this common disorder. Like the extensive folklore devoted to the subject, most of the publications were replete with uncritical considerations of a number of possible etiological factors and with accounts of uncontrolled and statistically deficient excursions into the field of prevention and cure. The one firm development had been the securing of evidence that the vestibular apparatus is essential for the production of motion sickness. And, subsidiary to this, there was the indication that in ships and boats linear rather than angular accelerations constitute the predominantly effective stimuli. Early in the War, when it became apparent that the moving of large numbers of men by sea and air to and through combat areas might be attended by high rates of motion sickness, the problem began to receive, for the first time, comprehensive experimental attention. Work done during the last seven years has clarified some of the mechanisms involved and has given assurance that a fair degree of control is possible. Much remains to be done. It is hoped that this presentation will stimulate further experimental attacks on a problem which contains many items of general physiological interest.

## GENERAL CONSIDERATIONS

*Symptoms.* The symptomatology of motion sickness is a matter which is important not only for the evaluation of prophylactic and curative measures, but also for

the standardization of procedures used in the experimental production of motion sickness. There are available a number of clinical descriptions of the condition (38, 43, 93, 99, 138). Some early students of the problem attempted to classify cases of seasickness on the basis of the doctrine of vagotonia and sympathicotonia (52, 99, 101, 139, 236). Thus they distinguished two forms of motion sickness, one in which signs of parasympathetic stimulation are predominant and one characterized by overactivity of the sympathetic system. The soundness of this classification, like the doctrine on which it is based, is most questionable, and recent investigators have discontinued it (5, 94, 175, 209).

Although the symptoms of motion sickness vary somewhat in number and intensity from individual to individual, it can be stated that on the average they are, approximately in the order of their appearance, the following: *drowsiness*, which may mark the transition from a somewhat pleasurable subjective response to motion to one which is suggestive of illness; *pallor*, chiefly facial; *cold sweating*, which appears to be the most reliable and constant indication of the onset of sickness; increased *salivation* with swallowing; the sensation of *nausea*, usually ushered in by some degree of epigastric awareness; and finally *vomiting*. The degree of incapacitation produced varies greatly and may bear little relation to the occurrence of emesis. Headache and 'dizziness' are vague symptoms which do not occur with regularity and cannot be used as specific criteria. It is noteworthy that nystagmus has rarely, if ever, been detected in seasickness and that close observers of the sickness evoked by the motions of airplanes, swings and elevators have failed to find it in their subjects. On the other hand, nystagmus and true vertigo do occur when sickness is produced by rotation.

Blood pressure and heart rate changes are insignificant during the development of seasickness and swing sickness. Some increase in pulmonary ventilation may occur; it is chiefly encountered in highly susceptible persons. No significant changes in blood composition have been found except as the results of other changes (alkalosis after hyperventilation; alkalosis and ketosis after long continued vomiting). Gastric hypotonia and hypomotility are often produced by motion; they occur more often among the susceptible than among the relatively immune, but they do not seem to be invariable accompaniments of motion sickness.

*Incidence.* The belief that susceptibility to seasickness is confined to a relatively small and special fraction of the population has been dispelled. It is commonly observed that in some individuals a given kind and intensity of motion may produce no symptoms or only mild ones, while in others it evokes severe symptoms. Also, depending on the individual's susceptibility, seasickness may develop within minutes or only after hours of exposure to motion. It appears that a person may be resistant to one type of motion but not to another (30, 61, 65, 95, 138, 149, 179). For example, some tolerate the motions of small boats but become sick on larger vessels, and vice versa. Certain individuals readily become sick on a swing but not in an airplane or ship, and Noble (171) has observed a similar phenomenon in dogs.

It was not until the exigencies of World War II evoked an urgent demand for factual information on this subject that data were collected which indicate with some precision the proportion of the population that is susceptible to motion sickness. It appears that almost every normal individual can be made sick by motion. There are, of course, many influencing factors.

Hemingway and E. L. Green (98) found that during the initial training of student aviators (College Flight Training Program) 11 per cent of 2689 students were sick on one or more of their first 10 flights. The same incidence was found by Rubin (197) in a study of 837 cadets in a primary training detachment. Mathewson (146) reported that of 2682 air crew trainees 15 per cent were sick at least once during their training. McDonough (149) observed that 65 per cent of 380 navigation cadets reported being airsick one or more times during 4534 missions. Similarly, in a study of 1006 experienced flying personnel who were followed after they joined a combat bomber crew training unit, D. M. Green (84) found that during their training 52 per cent of 176 navigator-bombardiers suffered from sickness on one or more occasions and that 23 per cent were sick five or more times. The sickness rates shown by the other categories were: radio-gunners, 32 per cent; navi-bombardiers, 19 per cent; engineer-gunners, 16 per cent; pilots, 13 per cent; armorer-gunners, 11 per cent. The average incidence was 17 per cent. Australian investigators (157) found the incidence of airsickness in navigator trainees to be 33 per cent; persistent sickness occurred in 10 per cent of these subjects. In comparing studies on individuals undergoing primary training with those on navigators, gunners, radio operators and bombardiers, one must bear in mind that during the initial training period the total flying time is short and exposure to rough flying conditions is apt to be avoided because of the inexperience of the students. Although the higher incidence among navigators, bombardiers, gunners and radio operators may have been due in part to the fact that some of these men had been eliminated from pilot training because of airsickness, it is true that their duties require conditions of vision and posture which tend to facilitate the development of motion sickness (see below).

The incidence of sickness has often been reported in terms of 'man missions,' that is, the amount of sickness found in, say, 50 men during 100 flights (5000 man missions). Hemingway and E. L. Green (98) reported 661 cases of airsickness among 2689 men, each of whom took 10 flights, an incidence by flights of approximately 2.5 per cent. Brown, Brett and Howlett (45) found an incidence of 8.3 per cent during 2775 manflights by 91 navigation students. On the basis of man missions, McDonough (149) reported an incidence of 15.6 per cent among 380 navigation cadets who flew 4534 missions. In a Navy study (127) an incidence of 7.5 per cent was found in 531 cadet flights. When the trips were rough, as was the case in 205 of these man missions, the incidence was 25 per cent. The practice of reporting sickness rates on the basis of man missions demonstrates the magnitude of the problem for the armed services, but it masks the true situation, since the effects of adaptation and the influence of different degrees of roughness during different flights are not indicated.

In an airborne maneuver involving 10,070 men on a flight of 130 to 150 miles under ideal flying conditions the incidence was 3.56 per cent (147). In a study (131) of the effects of preventives during airborne maneuvers it was found that the incidence of sickness among the untreated controls varied in general with the length of the flight; it was concluded that "in flights exceeding two and one-half hours and performed under normally-expected variations of flying conditions, more than one quarter of the passengers may be expected to become airsick." Littauer (168) reported an incidence of 80 per cent in a five-hour flight involving airborne troops in planes and gliders. Park (180) found an average incidence of 35 per cent during glider maneu-



ers. In an experimental study (221) of airsickness, 65 unselected but experienced parachutists were exposed to 'moderate evasive tactics.' After 20 minutes all 65 subjects, together with 4 observers and 2 radio operators, were airsick.

As regards seasickness, Hill (99) estimated that over 90 per cent of inexperienced persons will become ill under severe conditions. The correctness of this estimate is borne out by more recent studies. British investigators reported incidences of seasickness in small boats ranging from 15 to 70 per cent (103, 105, 107). In tests of the efficacy of pharmacological preventives Tyler (220, 221) obtained data on sickness rates in small landing craft during amphibious training operations which lasted from one to three hours. In the 36 groups, totaling 3133 unselected young men, which received placebos (lactose in capsules) the rates varied from 11 per cent, when there were gentle swells, to 60 per cent, when the seas were moderately rough. In the former instance only one-fifth of those affected were severely sick (severe nausea with or without vomiting), in the latter, one-half. Since, in the course of these same observations, it was found that the giving of a placebo did not affect the incidence or severity of sickness, these rates may be regarded as indicative of the general level of susceptibility to the kinds and degrees of motion experienced. They suggest that unofficial reports of rates approaching 100 per cent in certain combat landing operations during the War were not exaggerated.

In many experimental studies of motion sickness, swings have been used to test susceptibility and the effects of drugs and to explore other aspects of the problem. Parker, Fields and Sellers (184) reported that 36.4 per cent of 2950 men showed symptoms during a swing test of 30 minutes. Hemingway (94) found that of a group of 489 normal young adults exposed to the motion of a swing for 20 minutes (or less, if vomiting occurred) 29 per cent became sick. In a study (95) of 1500 men in the Army Air Forces, whose airsickness histories were known, this brief swing test gave vomiting rates which varied from 11.3 per cent for flyers who had never been airsick to 65.4 per cent for airsickness eliminees. Noble (173), using a somewhat more severe swing test, obtained rates of vomiting as high as 56.6 per cent.

*Individual Predisposition and Adaptation.* It has been pointed out above—and it is common knowledge—that there are great variations among individuals in susceptibility to motion sickness. Failure to control this factor in experimental studies has resulted in some errors. In such work it is essential to use either carefully selected subjects of known susceptibility or, where this is not possible, large heterogeneous groups randomly divided into the necessary number of sub-groups.

It has been stated that children under 2 years of age and elderly persons show less susceptibility than those of intermediate age (30, 38, 43, 44, 61, 114, 178). Some indication was obtained by Noble (173) that susceptibility to swing sickness increases after the age of 40. It has been held, though on the basis of scanty evidence, that the incidence is higher in females than in males, and that there are differences between races (38, 43, 44, 54, 178). Many deaf-mutes, and persons with internal ear damage are immune (117, 196, 162, 205, 47). It has long been held that 'neurotics' are more prone to motion sickness than 'stable' individuals, but there is actually very little evidence to support this rather popular view (see below). A number of writers (60, 71, 79, 99, 176) have claimed that individuals characterized by an abnormal excitabil-

ity of the parasympathetic system (the 'vagotonia' of Eppinger and Hess) are particularly susceptible. Some apparently favorable results obtained with atropine medication (together with a disregard of the central effects of this drug) constituted the principal support for this idea.

There is evidence that horses, dogs, cows, sheep and chickens are subject to motion sickness (52, 73), and that rabbits, guinea pigs and pigeons are immune (52, 121, 192, 218). Dogs show degrees of susceptibility that are about the same as those displayed by the human race (170, 171), but cats and monkeys are highly resistant (22, 164, 171).

A good many attempts have been made to discover the physiological or psychological peculiarities of an individual which determine his degree of susceptibility to motion sickness. Actually little has been learned of the basic factors which underlie vulnerability. Scattered through the pages that follow are references to a number of opinions, suggestions and a few experimental facts which bear on this important aspect of the problem. By way of summary about all that can be said is that susceptibility appears to depend on a rather specific constitutional capacity to respond to certain patterns of vestibular stimulation and that it can be modified to some extent by several extralabyrinthine influences.

It is estimated that about 95 per cent of all susceptible persons are capable of adaptation to motion. Hemingway (97), in a study of 198 aviation students, found a progressive decrease in the incidence of airsickness during the training period. Eighty-four per cent were sick during the first flights, only 10.5 per cent on the 10th flight; the decrease was more rapid in the first than the second 5 flights. Joeke (120) reported that while the incidence of airsickness was 17 per cent during the first few hours of flying it dwindled to 0.5 per cent after 10 hours of experience in the air. Adaptation also occurs on swings. In a study of 254 air gunners, Joeke found that although the incidence of sickness during a first swing session was 20 per cent, only 2.8 per cent of those who were sick failed to adapt during subsequent swinging. Manning (141) divided 100 unselected subjects into two groups. The members of one group were swung for 15 minutes on each of 10 successive days; the members of the other group were not swung until the 11th day. On the 11th day the first group showed an incidence of 18 per cent, the second one of 42 per cent.

In spite of some contradictory reports, the evidence available indicates that adaptation to one type of movement does not necessarily adapt the individual to the motion produced by a different vessel, airplane or experimental device. Gibson Manning and Cohen (81), in a study of 150 student air navigators which involved 4000 swing sessions and 1250 flights, found that daily exposures of 15 minutes to the motion of a swing for two weeks before and during the flying period did not reduce the amount of airsickness.

It appears that the capacity to adapt is minimal or entirely absent in those highly susceptible individuals who make up perhaps 3 to 5 per cent of the population. Brown, McArdle and Magladery (47) secured evidence that while some very susceptible subjects develop an increased tolerance when repeatedly exposed to a swinging motion, such adaptation does not occur in chronically sick individuals. Morton and McEachern (165, 166) attempted to produce adaptation in a very susceptible

flyer who had been grounded because of recurring airsickness. As a result of daily exposures to motion for 23 days there developed only a small degree of tolerance; it was insufficient to enable him to return to flying duty.<sup>1</sup>

*Experimental Methods of Producing Motion Sickness.* The means which have been effectively used to produce motion sickness experimentally are: *a*) devices which reproduce artificially the movements of a ship; *b*) elevators or elevator-like machines; *c*) swings; *d*) apparatus which subjects an individual simultaneously to rotation and tilting movements of the head.

Quix (195) cites the early work of Kreidl, who apparently produced motion sickness in animals by artificial ship movements. Pozerski (191) designed a see-saw-like machine that simulated the pitch and roll of a ship. In his extensive studies Sjöberg (204, 205) first used ordinary passenger elevators (lifts). When he found that "strong acceleration and a large amplitude" were required to produce symptoms quickly and easily he constructed a crane. The 'Roll-Pitch Rocker' constructed at the Montreal Neurological Institute in 1942 (56, 165) and the similar but smaller machine built at the Banting and Best Department of Medical Research in Toronto were designed to reproduce the movements of a ship at sea. Although they proved effective they later gave way to simple swings which permitted the ready testing of larger numbers of subjects. Wollaston (232), who was apparently one of the first to try the swing in a study of motion sickness, stated as early as 1810 that "the sickness occasioned by swinging is evidently from the same causes as sea-sickness. . . ." The first to use the swing extensively for experimental purposes were workers in Russia (234, 235) and Brown, McArdle and Magladery (47) in England. It was soon adopted by various workers in Canada, Australia and the United States. An advantage of the swing and also of the elevator designed by Wendt (5, 225) is that, since the various characteristics of the motion can be controlled, large numbers of subjects can be exposed to identical conditions of motion. The types of swings generally employed are the 2-pole and the 4-pole; both may be operated manually or electrically.

The 'wave machine' of Wendt (5, 225) is a hydraulically driven elevator with an air-tight, sound-proof, temperature-controlled cab, which operates in a shaft allowing vertical excursions up to 18 feet. It is essentially a vertical accelerator in which velocity, acceleration level and amplitude can be varied to give a variety of wave forms. It was designed primarily for the study of the characteristics of a motion which make it nauseating. It has proved capable of inducing severe sickness.

Many workers, impressed by the similarity between disturbances produced by rotatory movements and those produced by moving vessels, have employed rotating

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<sup>1</sup> There is evidence that, as regards adaptation, dogs are quite like human beings. A few instances of this phenomenon were encountered by one of us (26) in dogs which were subjected, at weekly intervals, to the motion of a swing, but the only substantial study of adaptation in animals of which we are aware came to our notice while this review was still in progress. Professor R. L. Noble of the University of Western Ontario very kindly provided us with a copy of the manuscript of his paper entitled "Adaptation to Experimental Motion Sickness in Dogs" (170a). This report makes it clear that adaptation is produced readily in animals having a relatively low initial susceptibility, but not easily in highly susceptible dogs. The rate of the development of the refractory state appears to be proportional to the frequency of exposure and perhaps to the duration of the individual exposures. It is interesting to note that adaptation occurred even though the animal was effectively treated by the thiobarbiturate, V-12.

chairs, tables and the like. For the production of sickness the subject must be exposed to repeated accelerations and decelerations. When an individual is rotated in the usual way on a Bárány chair changes in velocity occur only at the beginning and the end of rotation. As will be disclosed below, the essential repetitive changes in velocity exert their effects through non-auditory labyrinthine receptors. When rotation occurs with the head fixed, the spatial relationship of the various parts of the labyrinth to the axis of rotation remains constant, a fact which has been emphasized by Spiegel (216). Actually this type of motion is not very effective in producing sickness. Symptoms of much greater intensity have been obtained by changing the position of the head during rotation. For an experimental study of motion sickness, Spiegel, *et al.* (216) devised a chair in which, with each turn, the head of the subject is tilted either in the sagittal or the frontal plane. Thus the angle between the plane of rotation (horizontal) and the plane of the horizontal or vertical semicircular canals is made to increase and decrease with each rotation. This exerts the same effect on the canals as would repetitive changes in velocity. The apparatus proved to be very effective in producing sickness. Spiegel *et al.* believe that this device subjects the labyrinth to a type of stimulation which is essentially the same as that provided by other effective forms of motion. It is certain, however, that its effects on the labyrinth as a whole and on the utricular maculae in particular are quite different from those produced by a swing, an elevator, a plane or a vessel. The emphasis in the work with this apparatus is on stimulation of the cristae, but the authors suggest that stimulation of the maculae may play a role in the genesis of the sickness obtained.

#### ETIOLOGY

It was the opinion of Hippocrates that when a draught of hellebore had been given to provoke emesis the patient "should be made to move more about, and indulge less in sleep and repose" (*Aphorisms, IV, 14*; ref. 3). He supported this precept by stating that "sailing on the sea shows that motion disorders the body." It is evident that the Father of Medicine believed that seasickness is caused by the movements of the ship. Some recent writers, especially those with psychiatric interests, have not agreed with this view and have been inclined to attribute both seasickness and airsickness to other disturbances than motion. The occasional failure to appreciate the fact that the fundamental cause of motion sickness is motion has led to some confusion and conflict of ideas. It is perhaps explained, at least in part, by the fact that a stimulation of labyrinthine receptors, adequate to evoke motion sickness, produces little effect on consciousness. As Hemingway (96) has pointed out, "the patient is sick without being aware of the cause" and therefore is apt to attribute his troubles to odors, overheated compartments, visceral sensations, disagreeable sights, distasteful food or anything else unpleasant, unusual or uncomfortable which he experienced shortly before or simultaneously with exposure to motion. Unfortunately some observers have adopted one or another of these interpretations of their subjects.

*Characteristics of Effective Motions.* From a logical point of view the first step in an analysis of the cause or causes of motion sickness should be the determination

of those characteristics of motions which make them nauseating. Common observation suggests and experiment demonstrates that an essential feature of an effective motion is a repetitive change in velocity.

One group of early students of the problem, influenced by knowledge of the nauseous effects of rotatory movements, were inclined to attribute seasickness to angular accelerations in the movements of ships. Others made a careful examination of these movements and concluded that in the production of *mal de mer* linear accelerations in the vertical plane are prepotent. A majority of both groups, aware that destruction of the labyrinths abolishes all susceptibility to motion sickness, were primarily interested in applying their conclusions to the resolution of the question whether the receptors involved are the otolith organs or the cristae of the semi-circular canals (see below). These earlier studies did not contribute any precise information concerning the essential features of effective movements. Only recently, as a product of the widespread interest in motion sickness engendered by the events of World War II, has some definite knowledge of this aspect of the problem been secured. The chief results have been obtained by the experimental determination of the effects of altering certain characteristics of the motions of swings and vertical accelerators.

Cipriani (55) demonstrated that when a subject is seated upright on a swing, radial acceleration acting in the plane of the long axis of the body is the principal vestibular stimulus. He points out that swinging also involves three main components of tangential acceleration. The largest, that relative to objects around the swing, affects equally the mobile and the fixed portions of the vestibular apparatus and therefore cannot serve as a stimulus. He describes two other tangential accelerations which may be slightly effective stimuli. It is of interest to note that the  $g$  changes acting on the head of an individual subjected to the motion of swings used in experimental studies of motion sickness are of approximately the same order of magnitude as those which occur in the vertical direction in a certain type of plane (Anson) flying in moderately rough air (46).

In the case of a swing the magnitude of the maximal change in  $g$  depends on the arc; as long as the angle through which the swing oscillates remains constant, a change in radius does not alter the magnitude of the radial (linear) acceleration. On the other hand, the radius determines the frequency of oscillation and the duration of the change in velocity; the shorter the distance from the fulcrum, the greater the frequency. Thus if the arc be kept constant one may vary the frequency of occurrence of the same  $g$  change by altering the radius.

The effects of altering either frequency or maximal change in  $g$ , while keeping the other factor constant, were investigated by Fraser and Manning (78) in a study of 250 unselected normal individuals (aircrew personnel at an Initial Training School). Each subject was swung for 30 minutes unless extreme nausea or vomiting or both necessitated stopping before that time. In one series of experiments the rate of swinging was kept constant at 17 per minute while the maximal change in  $g$  was varied by changing the arc. With a maximal change of 0.25  $g$  the incidence of sickness was 22 per cent; increasing the  $g$  change to 0.9 more than doubled the sickness rate (50%), but a further increase to 1.7  $g$  resulted in an incidence of 46 per cent. Somewhat simi-

lar results were obtained by Noble (171) in experiments on dogs. Using a constant radius giving a frequency of 14.5 per minute he found that arcs of  $22.5^\circ$ ,  $45^\circ$ , and  $90^\circ$  produced sickness rates of 37, 90 and 100 per cent, respectively. These two sets of results suggest that increasing the  $g$  change increases the effectiveness of the motion up to a certain point beyond which further increases fail to augment the incidence. Changes in the frequency of a swing appear to exert similar effects. When Fraser and Manning (78) kept the  $g$  change constant at 0.9 and varied the radius they found that although a decrease in frequency from 22 to 17 per minute increased the incidence from 4 to 50 per cent, a further slowing to 15 merely raised the sickness rate to 58 per cent. In his dog experiments Noble secured evidence that for any given angle of swinging there is an optimum frequency (radius). Increasing or decreasing the frequency caused a falling off in the incidence of vomiting. The only exception occurred when the arc was small ( $22.5^\circ$ ); then the sickness rate showed a second rise when the frequency was greatly augmented by making the radius very short (37.5 inches).

The motion of a swing is made up of a vertical, a horizontal and a small angular component. Noble (171) separated these components and subjected dogs of known susceptibility to each. The horizontal proved to be the most effective, the vertical induced vomiting only in the more susceptible animals, and the angular failed to provoke any signs of sickness in the most susceptible dogs. It is significant that the composite motion was found to be more effective than any one of its components. McIntyre (156), who used human subjects, found that the repetitive up-and-down motions of an elevator, with accelerations only in the vertical plane, produce less sickness than do swings which yield comparable  $g$  values (see also 142). He also found that while the angular components of the motion of a swing are by themselves not nauseating, the addition of small angular components to movements in the vertical plane greatly increases the effectiveness of the latter. Thus we are faced with the probability that the repetition of a combination of linear accelerations in different planes or of linear and angular accelerations may constitute a more potent stimulus than the repetition of any one acceleration.

Work done by Wendt and his colleagues (5-11) constitutes the most extensive attack yet made on the problem of determining those features of a motion which make it nauseating. These investigators used the 'wave-machine' described above and thus confined their analysis to movements in the vertical plane. Their subjects were 477 Naval Aviation cadets. Exposure was for 20 minutes unless vomiting occurred earlier. The subjects sat blindfolded, with head in the upright position and with the cab temperature at  $86^\circ$  F. Evidence of the careful design of the experiments will be found in the original papers.

Wendt was led to examine the effects of varying the time interval between accelerations by the common observation that vehicles and movements which yield large accelerations with brief phases are rarely nauseating whereas those having low accelerations and long phases are apt to be very productive of nausea and vomiting. The first test (5) of the hypothesis thus suggested was a comparison of the effects of four waves in which the interval between equal accelerations at top and bottom was made to vary by changing the duration of a period of constant velocity (400 ft/min.) inserted in the middle of each wave. In the different waves this period was 0.22,

0.68, 1.12 and 1.6 seconds, the cycles per minute, 32, 22, 16, and 13, and the amplitudes, 4, 7, 10 and 13 feet. As the temporal separation of the accelerations was increased the subject was exposed to fewer and fewer of them and less total work was done on him. The 16-cycle wave with only half the rate of power expenditure but twice the duration of the 32-cycle wave produced six times as much sickness. The other waves were intermediate in effectiveness. Evidently the wave of longest duration (13 cycles) represents a recession of the optimum condition. These results confirmed "the hypothesis that the time characteristic of a motion rather than its violence is the feature relevant to motion sickness."

A second investigation (6) of the same hypothesis dealt with the question whether the significant variable is total wave duration or duration of the period between accelerations. Three wave types were obtained by changing not only the period of constant velocity in the middle of the wave but also the velocity itself (the greater the duration the less the velocity) while holding the wave cycle duration constant at approximately 22 cycles per minute. Peak values of acceleration were held constant. The intervals between accelerations were varied by varying the length of time of the application of the acceleration. The total energy per cycle (measured by peak velocity) increased as the interval between accelerations decreased. It was found that an increase in time between accelerations from 0.68 to 1.12 seconds with a reduction in total energy per cycle, resulted in a significant decrease in sickness. It was therefore concluded that when total wave duration is held constant, the total energy per wave is a more potent factor than is the interval between accelerations.

A third study (7) was devoted to a determination of the effects of acceleration level. Four wave types were obtained by controlling the rate of change of velocity and so altering the frequency and the total wave amplitude (between extremes of 4 and 9 ft.). The total energy per wave was kept at a constant value (mid-wave velocity of 400 ft/min.). The fastest wave with the highest acceleration level (32 cycles, 0.65 g) produced the least sickness (13%) while a wave of moderate frequency and moderate acceleration (22 cycles, 0.36 g) gave maximum sickness (53%). Two slower waves at lower acceleration levels (16 cycles, 0.25 g; and 13 cycles, 0.20 g) were somewhat less effective (rates of 43 and 40%). The results showed that nausea and vomiting are most effectively evoked by waves of moderate frequency and acceleration level. As regards the effects of frequency and g value they are in accord with those obtained by Fraser and Manning (78) in a study of the characteristics of the motion of a swing which make it nauseating.

The purpose of a fourth study by Wendt and his collaborators (8) was to find whether sickness production is a function of wave frequency or of duration or magnitude of component accelerations. Five waves were used, all having the same total energy. One, like the waves previously described, was a symmetrical acceleration wave. Four were asymmetrical; the amplitude, duration and g value of the acceleration differed in the top and bottom halves. The cycle duration of two of the asymmetrical waves was the same as that of the sickness-producing symmetrical wave used, while that of the other two was almost identical with the duration of the most nauseating wave of the third study. Consequently all the asymmetrical waves had intervals between accelerations which might be expected to induce high sickness rates

if cycle frequency is important. The top and bottom halves of the unequal waves had different acceleration levels. In two the different  $g$  values were ones which when used in symmetrical waves were relatively non-nauseating. Each of the other two was composed of a relatively ineffective half-wave and a nauseating half-wave. The sickness rates obtained were very close to what would be expected if the half-wave character rather than wave frequency determined the result. The authors indicate that this conclusion infers that effective motion acts on some resonant mechanical system with a natural period of about 3 or 4 seconds, but they point out that all structures in the body likely to be involved are short-period, heavily damped systems. They recommend therefore that acceptance of the conclusion be deferred until further evidence is forthcoming.

*Vestibular Factors*<sup>2</sup>. The idea that motion sickness is due to vestibular stimulation probably had its origin shortly after Goltz (1870), and Breuer, Mach and Crum Brown (1873-74), by elaborating the much earlier observations of Purkinje (1820-25) and Flourens (1828), showed that motion evokes responses through activation of labyrinthine receptors. According to Sjöberg (205), Irwin and Palasne de Champeaux were probably the first (1881) to call attention to the similarity between seasickness and Menière's disease and to suggest that the former is caused by stimulation of the labyrinth. This conjecture gained support from early observations which indicated that many deaf-mutes and others with internal ear damage are immune to seasickness. In 1882 William James (117) reported that approximately one-third of 519 deaf-mutes he had studied were not made dizzy by rotation, and a little later Pollack (188) found that he was unable to provoke vertigo by galvanic vestibular stimulation in 30 per cent of the deaf-mutes he examined. A majority of the refractory subjects studied by James suffered from acquired deaf-mutism. Fifteen of them had at one time or another been exposed to rough weather at sea, but not one had been sick. A few years later Minor (162) mentioned observations made at sea which indicated that many deaf-mutes are not susceptible, and Reynolds (196) pointed out that persons with internal ear damage produced by otorrhea are immune to seasickness. Much later Sjöberg (205) reported that 3 girls from a school for the deaf, who showed no responses to severe caloric and rotational vestibular stimulation, were not made ill by exposures of 2 hours to the motion of an elevator, although this same stimulus caused sickness within 30 minutes in most normal individuals. Still more recently Brown, McArdle and Magladery (47) found that 5 patients, whose labyrinths had been destroyed by meningitis, neither exhibited nor experienced any sign of sickness during 40 minutes of swinging.

Quix (195) states that Kreidl was the first to produce experimental motion sickness in animals; he exposed them to artificial ship's movements. This worker, according to several writers (54, 61, 193, 195, 205) found that after bilateral section of the eighth nerves the animals were insensitive to motion. Since Kreidl apparently

<sup>2</sup> In 1942 McNally and Stuart (159) published an excellent review of the physiology of the labyrinth which set forth all available relevant information that might be useful to the student of motion sickness. In this section we have drawn heavily on their presentation, but we have sought to limit our considerations of the functions of the non-auditory labyrinth to those items which seem to bear most directly on the problem of the etiology of motion sickness. We include a number of experimental facts and certain theoretical points which have been put forward since 1942.



never published the results of these experiments, the credit for first proving experimentally the indispensability of the labyrinths in the production of motion sickness goes to Sjöberg whose classical work reported briefly in 1929 (204) and fully in 1931 (205) demonstrated that bilateral labyrinthectomy renders susceptible dogs wholly immune to motion sickness. Before the operation his 4 dogs had shown marked symptoms and had vomited after 11, 12, 20 and 30 minutes when exposed to the motion of a crane. After the destruction of the labyrinths they failed to develop any sign or symptom of motion sickness during exposures of 3 hours. Recently McNally, Stuart and Morton (160) and Babkin and Bornstein (20) have confirmed this important discovery.

A question which has been and remains controversial is whether one or more than one of the sense organs of the non-auditory labyrinth discharge the afferent impulses which are essential for the production of the symptoms of motion sickness. There are three schools of thought on this question: One believes that both groups of receptors, the cristae of the semicircular canals and the maculae of the otolith organs, are involved; another school maintains that only the maculae are concerned; a third group holds that the otolith organs are not essentially involved and that motion sickness is caused chiefly, if not solely, by stimulation of the cristae.

It appears that the only labyrinthine sense organs to be considered in this connection are the cristae of the semicircular canals and the utricular maculae. Although the saccule has been considered by many to be part of the vestibular mechanism, the observations of a number of workers strongly indicate that it is not concerned in the elicitation of any known labyrinthine static or kinetic reflex but may be responsive to vibrations. (A full and convincing discussion of this work is given by McNally and Stuart, 159.) Thus a considerable weight of evidence seems to eliminate the saccular maculae as receptors involved in the genesis of motion sickness.

Although Brown (48), Magnus and de Kleyn (137) and de Kleyn and Versteegh (123) believed that linear and progressive movements stimulate the semicircular canals, it is now the consensus of opinion that the utricular maculae are the receptors chiefly stimulated by linear accelerations and that the sense organs of the canals respond chiefly to angular accelerations. However, there is some evidence that the utricles can be stimulated by very rapid angular rotations (159). Nystagmus is a result of stimulation of the cristae. Neither stimulation nor injury of the utricle produces it (88, 137, 223, 224). A depressor response to rotation can be evoked after the cristae have been made inexcitable by puncture of the round window, but it can also be evoked after all known reflexes of utricular origin have been paralyzed by the centrifugation method, provided the semicircular canals remain functionally intact (129). It has been concluded from such studies that the non-auditory labyrinth functions as a whole and that its two types of receptors can substitute for one another.

Many of the earlier workers (1, 2, 24, 50-52, 138, 199), impressed by the similarity between the symptoms experimentally produced by angular accelerations and those of seasickness, arrived at the conclusion that the semicircular canals are partly, or even wholly, responsible for the production of seasickness. Bruns (50, 51) found that persons who were highly susceptible at sea were easily made sick by rotary movements, while those who were immune on shipboard were relatively insensitive to the nauseating effect of rotation. Fischer (72, 73) also believed that the semicircular

canals are responsible, since in his experiments typical nausea was produced by rotation. He held that stimulation of the maculae is not necessarily involved in the genesis of seasickness. He evoked severe symptoms by rotating his subjects in a chair and simultaneously having them move their heads with an even rolling motion so that the position of the head was constantly changing in relation to the axis of rotation. He reported that under these conditions there was no distinct nystagmus. Spiegel and his associates (216) obtained high rates of sickness by means of their rotating-tilting machine (see above). In these experiments nystagmus always occurred. Almost all observers agree that nystagmus is not observed in seasickness, airsickness and swing sickness (42, 63, 108, 138, 169, 194, 205, 229). Humphreys (114), however, in listing the symptoms of seasickness stated that "temporary nystagmus has even been observed," but he gives no further information on the point. In considering the sickness produced by angular accelerations it should be borne in mind that several careful studies have failed to establish any good correlation between responses obtained in the Bárány chair and susceptibility to airsickness or swing sickness (see below).

The absence of nystagmus in seasickness has been emphasized by many as an indication that the receptors of the semicircular canals are not stimulated by the movements of ships and hence are not concerned in the genesis of seasickness. Wojatschek (229) concluded that angular accelerations do not, in the case of a rolling ship, have an effect because the phases of motion succeed one another rapidly and so annul one another. Sjöberg (205), who concluded that in seasickness both the semicircular canal system and the otolith apparatus are effectively stimulated, suggested that the absence of nystagmus is due to stimulation of both labyrinths in such a way that the impulses from one side counteract the effects of those from the other side and therefore no observable eye movements occur. But since Fleisch (76) had reported that in rabbits small reflex eye movements, not detectable by the naked eye, can be evoked by horizontal linear acceleration, Sjöberg thought it highly probable that similar responses, not observable by ordinary inspection, occur during experimental exposures to linear accelerations in the vertical plane and on shipboard when the waves are high. Maitland (138) contended that the absence of nystagmus in seasickness can be explained on the basis of the time periods of the various movements of the ship, particularly the pitch, which is the most nauseating. With Major Reed he determined that one phase of this movement rarely exceeds 5 seconds, which is only one-third of the time taken by the 8 rotations of the chair test. Further, he claimed that the pitch acts on the vertical canals rather than the horizontal. Therefore the comparison should be made between the effect of the ship's motion and a chair test of three continuous rotations which act on the vertical canals, a stimulus which is unlikely to elicit the primary reactions such as nystagmus. Spiegel and his co-workers (216) hold that nystagmus is not produced in the seasick because the rolling movements of a ship have a relatively small amplitude, each being in the direction opposite that of the preceding one, and are not complete rotations through 360 degrees in any one direction. According to these authors this results in only small oscillations of the cupola insufficient to cause ocular reflexes, but strong enough to produce 'vestibulo-vegetative' reactions.

Indirect evidence in support of the theory that the utricular receptors are the

sense organs involved in the production of seasickness has been derived from studies of the types of movements produced by ships. It has often been stated that the vertical movements of a ship are more distressing than the rolling movements. Wilks (226) first pointed out the similarity of the sensations produced by the pitch of a ship and those evoked by an elevator. Wojatschek (229), Maitland (138), Quix (195), Sjöberg (205) and others (44, 163) made extensive mathematical analyses of the movements of ships. On the basis of threshold values of labyrinthine stimulation by angular and linear accelerations all concluded that the linear (horizontal and vertical) rather than the rotatory or angular movements are the principal stimuli in the production of seasickness. As early as 1875 Mach (see 159) had calculated the thresholds for stimulation by both angular and linear (vertical) accelerations. He estimated that for the former it is 2 to 3° per second acting for about 15 seconds, for the latter about 0.01 g (approximately 10 cm/sec.). Although Quix (195) cites others who give different values for angular acceleration, Mach's figures are still considered by many as standard. Both Quix and Sjöberg concluded that linear accelerations produced by the movements of a ship are well above the threshold for stimulation of the maculae and are, therefore, responsible for seasickness. They found that the angular accelerations are well below the threshold of stimulation of the cristae. Wojatschek (229) on the other hand, determined that in the movements of a small boat with a roll of 60° (which is an extreme roll) the angular accelerations are above the threshold value, but he concluded that they are not important in the elicitation of sickness. According to Maitland (138) any statement that the rolling of a large vessel exceeds 30° should be received with caution. In view of the information now available concerning the characteristics of motions which make them nauseating (see above), it is evident that considerations of the mere threshold of simple reactions or sensations evoked by labyrinthine stimulation have little significance for this problem.

Several authors (17, 116, 153, 200, 205) refer to work done with a suspended cabin designed to eliminate angular accelerations in the movements of a ship. It is significant that passengers in such a cabin, still exposed to the vertical movements, become seasick. Also, it is now well established that motion sickness can be readily provoked by elevators and other devices which yield only vertical accelerations.

The recent discovery that the incidence of swing sickness is enormously affected by the position of the head, regardless of the position of the body, gives further emphasis to the role played by utricular stimulation in the etiology of motion sickness. Quix (195) pointed out that the position of these receptors is such that they would be particularly responsive to linear vertical motion. He suggested that differences in sensitivity to motion could be brought about by altering the head posture and hence the relation of the otolithic apparatus to the plane of linear motion. The results of studies of swing sickness made recently by Howlett and Brett led to an analysis (111) which has indicated that the utricular maculae are responsible for this form of motion sickness. This important aspect of the problem is further discussed below.

It is perfectly clear that motion sickness can be produced by either angular or linear accelerations. When it is evoked by the former, the semicircular canals appear to be primarily involved and nystagmus is observed; when it is produced by vertical

or other linear accelerations the utricles are stimulated and no perceptible nystagmus occurs.] In practically all cases of sickness due to the motions of ships, boats, airplanes, elevators and swings, linear accelerations appear to constitute the principal stimuli. It is to be emphasized, however, that when it is said that the effective accelerations produced by ships and airplanes are *principally linear*, this does not mean that angular accelerations are absent or wholly ineffective. It is well known that the most disturbing movement of a ship is the 'corkscrew' or 'wobble,' a combination of scending (vertical accelerations) with pitching and rolling (angular and linear accelerations combined). When the passengers of an airplane are exposed for a short time to this sort of composite motion, as when 'mild evasive tactics' are executed, extremely high rates of sickness result (221). In this connection the observations of Noble and of McIntyre (described above) are of significance. They indicate that the addition of small angular accelerations to a motion which has only linear changes in velocity augments its nauseating properties. Since it is most unlikely that the angular accelerations which were added could have stimulated the utricle, they probably acted through the semicircular canals.

*Visual Factors.* The idea that the eyes are involved in the genesis of motion sickness is an old one. Irwin (116) points out that in his *Zoönomia* (1794) Erasmus Darwin, whose grandson suffered so greatly from the motions of the 'Beagle,' expressed the view that visual disturbances constitute the principal cause of seasickness.

It seems to be fairly well established that, in the absence of any motion of the head and body, visual reception of motion may produce some of the visceral changes of motion sickness (58), nystagmus and loss of equilibrium (41) and even nausea and vomiting (153). In the 'witch's house' of amusement parks a visual influence, by seeming to accentuate a slight motion, frequently causes sickness (62, 153). Sjöberg (205) in exposing 4 dogs to the movements of his crane found that the onset of symptoms was somewhat delayed when the eyelids were sutured. It is known that blind individuals may become motion sick, but it has not been determined whether the incidence of sickness among the sightless is different from that found among normal individuals. It has been reported that the showing for more than 30 minutes of a motion picture taken from the subject's seat in a moving swing failed to produce sickness in a group of students seated in a classroom (165).

Although, as just indicated, visual stimuli may facilitate the production of sickness by motion or even provoke it in the absence of labyrinthine stimulation, visual influences are more apt to ameliorate the effect of motion. In many of the recent studies of the sickness produced by swings and elevators care was taken to control visual factors by blindfolding the subjects. The results of at least 4 series of experiments (135, 143, 144, 144a, 157) have shown that susceptibility to swing sickness is greater when vision is excluded, either by blindfolding or by enclosing the swing.

A common observation is that when aircraft pilots fly as passengers they tend to show an augmented susceptibility to motion sickness. It is held that an important factor in the production of this change is the absence of the visual orientation which is possible in the pilot's seat (19, 75, 84, 190). The relatively high incidence of air-sickness found among aerial navigators and radio operators (84, 91, 93) and of sea-sickness among troops crouching below the gunwales in landing craft (220, 221) also

appears to be due in large measure to loss of visual contact with the ground or the horizon.

Spiegel and his co-workers (216) in studying the responses of 30 individuals to the motions of their rotating-tilting machine found that the incidence of sickness tended to be less when the subject fixed his gaze on a lamp which participated in all the movements of the head than when he watched 3 stationary lamps placed around the rotating chair. It is not easy to relate this observation to those mentioned above, but attention may be called to the possibility that the very rapid and complex eye movements which must have attended the 'watching,' but not the 'fixation,' constituted a factor of importance.

*Role of Non-labyrinthine Proprioceptive Stimulation.* Stimuli belonging to this category may influence the production of motion sickness. Poppen (189), in discussing the concept that seasickness is a disability of equilibration, alluded to the fact that, after blindfolding, equilibrium is quite well preserved in an individual with non-functional labyrinths, while exclusion of vision in a tabetic causes serious disturbances of equilibrium. From these well known facts he concluded that somatic changes are important in the genesis of motion sickness. The evidence now at hand suggests that body movements and body position as such are of little significance in the production of motion sickness. To be sure, Sjöberg (205) found that encasing his dogs in plaster casts lengthened somewhat the time before the symptoms of motion sickness appeared, an observation which indicates the desirability of examining further the role played by non-labyrinthine proprioceptive stimulation in the genesis of motion sickness.

In 1931 Flack (74) provisionally concluded, as a result of observations made at sea, that "generally speaking, subjects in whom eye muscle imbalance is not induced or aggravated do not suffer from sea-sickness. . . ." Best and his co-workers (31) found a definite increase in ocular imbalance after swinging, especially in susceptible individuals. On the other hand, Howlett and Brett (109) have reported that ocular muscle balance was poorer in a non-air-sick group than in a group composed of persistently air-sick trainees. The available data do not seem sufficient to permit any conclusion regarding a possible relationship between heterophoria and susceptibility.

In the face of the evidence that head position (i.e., the position of the labyrinthine receptors), regardless of body position, is such a prepotent determinant of the incidence of motion sickness all extra-labyrinthine stimuli appear to fade into relative insignificance.

*Effect of Position.* Much light has been thrown on the problem of the etiology of motion sickness by studies of the effect of altering the position of the individual in relation to the motion or motions to which he is subjected.

Awareness that the recumbent posture has a beneficial effect on the symptoms of seasickness is as old as navigation itself. Contributors to the early medical literature on the subject gave many, and sometimes bizarre, reasons to account for this effect. The more reasonable writers, in those days, tried to explain the phenomenon on the basis of the effect the horizontal position might have in reducing gross movements of the heavy viscera with tugging on nerve plexuses. Authors who were adherents of the ideas of Wollaston (see below) attributed it to possible circulatory alterations. Even today there are those who offer these explanations or modified versions of them.

It appears that Wilks (226) was the first (1875) to suggest that the ease with which seasickness is produced in the vertical position (as compared to the horizontal) may be related to the vertical motion of the ship. He arrived at this conclusion by noting that symptoms similar to those of seasickness could be produced in passenger elevators (lifts), which were being introduced at about that time. Irwin (115), impressed by the early work of Purkinje, Breuer, Mach, Crum Brown and others, was the first to offer an explanation based on the effect the assumption of a horizontal posture has in altering the position of the head, and thus that of the labyrinth. The relation of the position of the head to the incidence of seasickness received attention in the writings of Byrne (52), who found that symptoms typical of seasickness are much more easily produced on a rotating table when the subject is sitting with his head in a central position as regards the axis of rotation than when he is in the supine position with the head in a more eccentric position. Bárány (25) found that he could alleviate the unpleasant sensations experienced on a scenic railway by flexing his head 90°. More recently McDonough (147) reported that during an airborne infantry maneuver simulated casualties carried supine on litters showed no sickness whatever while a like number of men exposed to the same flying conditions in the sitting position showed an incidence of 3.8 per cent.

Quix (194) was the first to ascribe the effect of altering the position of the head to a change in the relation of otolith organs to the plane of motion. He recommended that on shipboard the susceptible individual should lie down with head inclined slightly backwards. He pointed out that in this position the utricular otoliths lie on "the blind spot of the static sense," a spot where they are lower than the ciliate cells and so do not lie on them. He believed that in this position the saccular otoliths may be influenced by rolling movements but "the rolling angle is so small that its influence is of a very limited importance."

Tyler (220, 221) found that body position is an important factor. When, during transfer from ship to shore in small landing barges troops were made to crouch, the incidence was 30 per cent as compared with 11 per cent when they were allowed to stand. Since in both postures the head is in relatively the same position it was concluded that other factors were operating. Exclusion of visual orientation as a result of crouching was doubtless one factor. Furthermore, since it is necessary for the development of motion sickness that the sense organs of the labyrinth maintain for some length of time a particular pattern of afferent discharge, the relatively greater fixation of the head in the crouching position must be considered a factor favorable to the production of seasickness.

The most extensive studies of the role of head position are those of McIntyre (155), Manning and Stewart (143-144a, 217) and Howlett, Wardell and Brett (112, 113). Manning and Stewart (143), in a study of 825 men, found marked differences in the incidence of swing sickness due to variations in posture (position of the head). In the supine position with the eyes open 5 per cent of the subjects became ill, while in the sitting position with the eyes open sickness was increased to 27.5 per cent. The maximum susceptibility occurred in the sitting position with the swing covered (64%), and it was high in this position with eyes closed (51%) or covered with black-out goggles (57.5%). They attributed the postural effect to alterations of the position of the vertical canals and offered as further support for this contention

the fact that the prone position resulted in more sickness than the supine. They concluded that any head posture in which the vertical canals assume a nearly horizontal position is the least conducive to the production of swing sickness. However, as indicated presently, there is another explanation of the results. Stewart and Manning (217) also reported that on the 4-pole swing the least susceptible position is sitting with the head turned back  $90^\circ$  from the horizontal. McIntyre (155) obtained similar results. In a group of 25 subjects swung in the supine position with head normally inclined the incidence was 16 per cent; in 25 swung in the supine position with head raised the incidence increased to 40 per cent; a somewhat higher incidence, 52 per cent, occurred in the prone position with head extended. McIntyre, however, concluded on morphological grounds that "the sensory end organ mainly responsible for motion sickness appears to be the utricular otolith organ of the labyrinth."

In a carefully controlled series of experiments involving 790 tests on 320 subjects, Howlett, Wardill and Brett (112, 113) investigated the effects of a variety of body and head positions on the incidence of swing sickness. With the subjects blindfolded they found that, regardless of the position of the body, the assumption of different positions of the head in relation to the radial (linear) accelerations of the swinging motion greatly affected the sickness rate. They chose as a reference for the position of the head "the plane of lines joining each external auditory meatus to the lateral canthus of the eye of the same side." Each subject was swung for 30 minutes unless sickness occurred earlier. All but 20 of the subjects were unselected air crew trainees. When 100 of these men were swung in the sitting position with the reference plane parallel to the base and perpendicular to the radius of the swing the incidence of sickness was 61 per cent. In 50 men swung also in the sitting position but with head tilted backward so that the plane was perpendicular to the base none became sick. Similarly, no sickness occurred in 50 men swung in the supine position with occiput down (plane again perpendicular to base of swing). On the other hand, the incidence was 68 per cent in a group of 50 subjects swung in the supine position with neck so flexed that the reference plane was parallel to the base of the swing. Obviously these marked differences cannot be related to the position of the body; they are clearly related to definite positions of the head. That the effect operates in highly susceptible individuals was shown by results obtained with a group of 20 men who had been eliminated from training because of persistent airsickness. In the sitting position, with reference plane parallel to the base, all became sick. Yet when they were swung in the same body position but with head back (reference plane perpendicular to base of swing) none was sick.

According to Quix (194) the base of each utricular macula lies parallel to the reference line used by Howlett, Wardill and Brett. This consideration, together with the evidence that repeated accelerations in the vertical plane, which stimulate the utricles, are effectively nauseating, led Howlett and Brett (111) to propose a theory of the mechanism of utricular response to this motion. In elaborating the ideas of Quix they suggested that the effective force due to swinging motion acts on the utricle in such a way that, depending on the position of the head, the otolith exerts a force on the macula or parallel to it or away from it. The results of the experiments cited above suggested that "stimulation for motion sickness is greatest when the force

tends to move the otolith onto the membrane, least when parallel to the membrane and intermediate when away from the membrane." Now Quix (194) had pointed out that although the base of the utricular membrane is parallel to the reference plane adopted by Howlett, Wardill and Brett, its anterior tip is curved upward. If the utricle is the sense organ that responds to accelerations in the vertical plane, it should be possible to obtain differences in sickness rates when the  $g$  changes occur in a plane parallel to the base of the macula but perpendicular to its upturned anterior tip. It is significant that Howlett, Wardill and Brett found an incidence of 18 per cent when the subjects were in the prone position with reference line perpendicular to the base of the swing (otoliths forced toward tip of membrane) and no sickness when they were in the supine position with the line also perpendicular (otoliths forced away from tip of membrane). Thus the hypothesis of Howlett and Brett has received substantial support. In a personal communication, however, McIntyre (156) states that, although on the swing different head positions produce different sickness rates, the results obtained on a vertical accelerator (a spring elevator) do not follow predictions based on the results of the swing experiments. He is of the opinion that the alleviating effect of certain head positions is only evident with composite motions.

The rotating-tilting machine used by Spiegel and his co-workers (216), which has been described above, imparts to the labyrinth of the subject a motion in which the velocity is rapidly and regularly altered throughout rotation of the chair at constant velocity. This exerts effects on the endolymph of the canals and so affects the cristae. The tilting also produces rhythmic vertical movements of the head, but these are of small amplitude and it is perhaps doubtful that they are of any significance in the genesis of the sickness produced. The total effect of the combined motions upon the maculae is not easily determined. The incidence of sickness was not affected by tilting the body as well as the head. Tilting the head in the frontal plane was somewhat more effective than tilting it in the sagittal plane. The highest incidence (80.8%) was obtained when during rotation a succession of head movements in the sagittal plane was followed by a succession in the frontal plane.

Although the controversy as to which one or ones of the sense organs of the non-auditory labyrinth are essential for the production of motion sickness, particularly seasickness and airsickness, remains to be settled, the results of these studies of the effect of head position afford almost a superabundance of evidence that labyrinthine stimulation is the primary factor in the etiology of motion sickness.

*Central Nervous Mechanisms.* On exposure to an effective type of motion sense organs of the non-auditory labyrinth discharge repetitively in some particular pattern to the brain. The maintenance of this afferent bombardment for some length of time seems essential for the development of motion sickness. The subjective experience called nausea, the visceral changes mediated through autonomic efferent pathways and, finally, the integrated somatic nervous discharges to skeletal muscle which cause retching and vomiting all result from the activation of one or more central mechanisms by what appears to be a most striking instance of temporal summation of afferent impulses. It is likely that a delimitation and physiological characterization of these central processes would add substantially to our understanding of motion sickness. When one considers *a*) that the drugs which have proved useful in the pre-



vention of motion sickness (see below) exert pronounced central effects and b) that there is little, if any, reason to suppose that their peripheral actions contribute to their effectiveness, it is probable that an elucidation of the central aspects of this problem may lead to a more rational therapy. A number of experimental facts bearing on the location of the essential central machinery are now available.

The role played by the cerebral cortex has been examined by two groups of workers. In studies of swing sickness in dogs Bard and his collaborators (26, 27) found that the following cortical ablations do not significantly alter the susceptibility of these animals to the emetic action of motion: bilateral temporal lobectomy; removal of both frontal poles (containing the somatic motor and sensory areas); removal of all neocortex except both frontal poles; ablation of all cortex on one side and removal of all neocortex on the other except the frontal pole. These results indicate that if any one portion of the cerebral cortex is essential for the development of motion sickness in the dog it lies outside the temporal, occipital, parietal and frontal areas of the neocortex. The question whether motion sickness depends on the functional integrity of any part of the cerebral cortex can be answered satisfactorily only by determining the effects of complete decortication in susceptible animals. In any such study it is important to make sure that the general health and nutritive condition of the animals are maintained, for there is evidence that when these are impaired a normal dog shows a lessened sensitivity to motion (26). At the time of their report Babkin and Schachter (23) had studied one dog in which the cortex of both hemispheres had been totally or nearly totally removed. Although the brain had not yet been examined, the animal behaved entirely like a completely decorticate preparation. Before operation the dog vomited, on the average, 20 minutes after the beginning of swinging. During its decorticate career it was repeatedly swung for periods of 60 minutes or more and, with one exception, never displayed any sign of motion sickness except a little salivation. On a single occasion, 2.5 months after operation, vomiting occurred after 15 minutes on the swing, but 5 days later the dog withstood the motion for 130 minutes without showing more than some salivation and chop-licking. This experiment, while most suggestive, is perhaps not entirely conclusive.

In the course of a study, partially reported recently in abstract form (27), typical motion sickness was induced in a chronically *decerebrate* dog which survived the operation for 145 days and was in excellent condition during the test periods. Before operation it was swung 7 times at weekly intervals and vomited on each occasion (times to vomiting, 4-10 minutes). The animal was decerebrated by removal of a wedge of tissue made up of caudal diencephalon and rostral mesencephalon after exposure of tectum and thalamus by bilateral ablation of occipital cortex, hippocampus and geniculate bodies. Autopsy showed that the rostral face of the brain stem remaining caudally passed from the cranial borders of the superior colliculi to a level about 1 mm. rostral to the origin of the third nerves. On each of 9 swingings carried out at weekly intervals between the 26th and 81st postoperative days the dog salivated excessively and vomited (times to vomiting, 3-9 minutes). Between the 117th and 138th days almost identical results were obtained in a second series of 4 tests.

The fact that the best known suprasegmental representation of the vestibular receptors lies in the cerebellum led Bard and his collaborators (26, 27) to examine the possibility that this part of the brain is concerned in the production of motion sickness. A dog which, in 11 swings at weekly intervals, had invariably vomited within 8 to 25 minutes and had always shown profuse salivation with licking and swallowing, was subjected to uncomplicated removal of the entire cerebellum (confirmed by post-mortem histological examination of the brain) and then studied over a period of 17 months during which time it remained in excellent health. In each of the 15 postoperative tests on the swing (14 of 60 minutes, one of 2 hours) the animal failed to vomit and the small amount of salivation and licking observed was always associated with panting. An interval of 3 months between the 12th and 13th tests and one of 6 months between the 13th and 14th swingings served to demonstrate the improbability that the immunity was due to an augmented capacity to adapt to motion. Very similar results were obtained in a series of previously susceptible dogs after removal of nodulus, uvula and pyramis. These animals, however, showed mild salivation and some licking in the absence of panting. In control experiments it was found that ablation of all parts of the vermis between primary fissure and pyramis or removal of pyramis and a few folia of uvula failed to reduce in any way the sensitivity of the animals to motion. The conclusion was drawn that nodulus and uvula contain neural mechanisms which are prepotently involved in the genesis of motion sickness in dogs. Evidence from various sources indicates that uvula, nodulus and flocculi (possibly also lingula) comprise the 'vestibular' portion of the cerebellar cortex. The results of these experiments leave uncertain the role of lingula and flocculi in the genesis of motion sickness. The slight symptoms (salivation and licking) noted above may have been due to the functional integrity of these structures.

It is interesting to note that the dog rendered immune to motion by decerebellation showed a normal sensitivity to the emetic action of apomorphine (a drug which acts directly on the bulbar vomiting 'center') and that the animals in which a partial cerebellar removal had abolished the emetic action of motion occasionally vomited in their cages after eating. These facts show that the bulbar vomiting center lies 'downstream' from the specific central mechanisms which are essential for the vomiting of motion sickness. Further, they indicate that susceptibility to motion sickness is independent of susceptibility to certain other emesis-producing agents and conditions. Bard, Woolsey and Snider (26), in a study of a series of dogs representing all degrees of susceptibility, could not find any correlation between sensitivity to swinging and the threshold emetic dose of apomorphine determined by quick intravenous injection. Birren, Stormont and Pfeiffer (37), however, in studies of human subjects, obtained some indication that the reactions to apomorphine permit a rough differentiation between susceptibles and non-susceptibles.

Another approach to the neurological aspects of motion sickness has been made through electroencephalographic studies. Neither Jasper and Morton (119) nor Lindsley and Wendt (130) were able to secure any evidence of a correlation between the characteristics or deviations of the electroencephalograms of subjects and their susceptibility to motion as determined by histories. Thorner (219) studied the electroencephalographic records of 130 consecutive cases of airsickness. Thirty-

two of these men yielded records containing unusual features. Although marked correlations were found between such recordings and independently made psychiatric estimates, it was concluded that the electroencephalographic changes could not be used as a means of pre-selecting susceptible individuals.

*Physiological Status and Susceptibility.* The notion that susceptibility is connected with a state of 'vagotonia' or 'sympatheticotonia' or with some instability of the central control of the autonomic nervous system is an old one to which some reference has already been made. Recent work has shown, however, that susceptibles and non-susceptibles cannot be distinguished on the basis of high or low resting blood pressures or heart rates or by changes in these on exposure to motion (31, 57, 94, 110). Wendt and his co-workers (12, 13, 90) found no differences between highly susceptible and non-susceptible individuals as regards their autonomic responses to such procedures as the cold pressor test, breath holding, hyperventilation, body tilting and injections of epinephrine or mecholyl.

Several attempts to correlate susceptibility with a greater than normal reaction to acetylcholine or to an anticholinesterase have been made. Babkin and his collaborators (21, 22) reported that in dogs there is some relation between susceptibility to small doses of acetylcholine and susceptibility to swing sickness, and they also found that when non-susceptible dogs were pre-treated with small doses of prostigmine they showed symptoms of sickness when subjected to swinging. Best, Sellers and Stephenson (32) concluded from experiments on a small series of dogs that there is probably some relationship between susceptibility to acetylcholine and to swinging. Goehring and Schwab (83) found that the injection of 1 mg. of neostigmine evoked no appreciable effect in individuals who were non-susceptible or who proved to be capable of rapid adaptation to motion, but induced all the signs of motion sickness in members of a group of men hospitalized because of chronic seasickness. On the other hand, Birren, Stormont and Pfeiffer (37), in another Navy study of quite similar material, found that the responses to the administration of this same amount of neostigmine, given by the same route, completely failed to distinguish between personnel surveyed to shore duty because of chronic seasickness and unselected representatives of the general population. Manning (140), in a study of 14 persistently airsick and 31 non-airsick trainees, did not find any statistically significant differences in blood cholinesterase activity between the two groups.

Conditions in the gastro-intestinal tract do not appear to be of significance in the determination of susceptibility, unless of course they are ones which tend to evoke nausea and vomiting in the absence of motion. Contrary to a popular conception, the interval after meals does not appear to affect the incidence of motion sickness (9, 31, 143). Schwab (201) has commented on the high percentage of gastric, pyloric and duodenal abnormalities found in patients hospitalized because of chronic seasickness. Almost certainly these disturbances were the results of long-continued bouts of seasickness and had nothing to do with the original susceptibility of the men to motion. Results obtained by McDonough (148) and by McDonough and Schneider (151) in gastro-intestinal studies of immune and susceptible subjects (see below) appear to justify the conclusion that "there is no specific type of gastro-intestinal tract, either from an anatomic or functional standpoint, which is associated with motion sickness susceptibility" (151).

Hemingway (96) found no significant difference in the incidence of swing sickness in cold and warm environments (range, 0° to 40° C.). A similar result was obtained by Magladery (136) and by Manning and Stewart (143). In a study of motion sickness in dogs, Best and his co-workers (32) could obtain no evidence that susceptibility is related to the level of ionized calcium in the blood. They also reported that a high potassium or high sodium diet does not appreciably affect sensitivity to motion.

*Bodily Changes Evoked by Motion and Their Etiological Significance.* A number of bodily disturbances mechanically evoked by motion have been regarded as primary or contributing causes of the trouble. For example, it has been suggested that the motions of a ship cause gross movements of heavy abdominal viscera, probably with tugging on the mesentery or traction on nerve plexuses, and that these mechanical effects are important factors in the genesis of motion sickness (121, 124, 126, 177, 189). Consequently a number of authors have tried or advocated the application of abdominal supports in the control of seasickness (43, 61, 101, 126, 189) and airsickness (233). No one has provided conclusive evidence that this measure has any beneficial effect. Aiken, Hoffman and Howlett (4) found that the wearing of a modified visceroptosis belt did not produce any significant diminution of the incidence of airsickness in a group of air navigator trainees. Although Mainland (see 165) found that the position of the heavy viscera might change as much as 5 cm. as a result of tipping the body, no correlation could be found between susceptibility and lability of the viscera. Other pertinent considerations also lead to the conclusion that visceral displacement cannot be of any real significance in the production of motion sickness. Jolting movements such as those which occur in horseback riding or during certain strenuous forms of play do not evoke nausea and vomiting, while many swaying motions of far less violence are very apt to bring on sickness (5, 138). Howlett, Wardill and Brett (113), in discussing their demonstration that the incidence of swing sickness may vary from 0 to 90 per cent with different head positions, while the position of the body in relation to the motion remains the same, have very properly pointed out that visceral displacement cannot be a significant factor in the genesis of this disorder.

Some etiological importance has been attributed to changes in the distribution of blood induced mechanically by movements of a ship. More than 130 years ago Wollaston (232), impressed by the oscillations produced in the mercury of a barometer by the up and down movements of a ship, propounded the theory that the blood in the vessels behaved in a similar fashion and so caused an alternate engorgement and anemia of the brain. Even quite recently this idea has had its adherents. Pflanz (187) made observations which led him to think that motion induces rhythmic changes in cerebral blood flow which have etiological significance. Genée (80) applied a collar to improve the cerebral circulation and reported some seemingly beneficial results of the procedure. Nunn (177) suggested that engorgement of the splanchnic vascular bed occurs and may be productive of motion sickness. Biehl (33) declared that a very important factor in the development of seasickness is the induction of mechanical changes in those parts of the brain which appear to float in cisternal fluid. None of these hypotheses has been supported by any factual evidence. As regards the supposed mechanical effect of motion on reducing cerebral blood flow, it may be noted that Spiegel, Henny and Wycis (215) found that the slight reduction in flow during rota-

tion is chiefly brought about indirectly through reflex vasomotor changes in the systemic circulation as a result of labyrinthine stimulation. Fraser (77) found that mild anoxia, equivalent to that obtained by ascending to an altitude of 10,000 feet, did not significantly augment the incidence of swing sickness.

In many discussions of motion sickness attention has been directed to the visceral effects which can be provoked reflexly by labyrinthine stimulation. For a more complete account of the reflex effects of labyrinthine stimulation on autonomically innervated effectors reference is made to the review by Spiegel (212). Spiegel and Démétriades (213) showed that in rabbits caloric, galvanic or kinetic (rotatory) stimulation of the labyrinth causes a reflex fall in arterial pressure, that this depends on the integrity of the sympathetic outflow, chiefly the splanchnic nerves, and that the effect is mediated centrally at the bulbar level. This reaction can be evoked from the utricular maculae as well as from the cristae ampullares (129). Spiegel and Démétriades (214) found that caloric vestibular stimulation increases the pendular movements and tonus of the small intestine and that this is a reflex mediated by vagal efferents.

Turning to the more relevant subject of bodily changes found in actual studies of motion sickness, one encounters a considerable number of observations on visceral alterations. Among these are several studies of gastro-intestinal functions. In an investigation of the effect of swinging on the gastric motility of 3 dogs, Babkin and Bornstein (20) found that the motion lowered the tonus and arrested the movements (hunger contractions) of the fasting stomach in a relatively non-susceptible animal as well as in the 2 very sensitive ones. The 2 susceptible dogs were later labyrinthectomized. After recovery from the immediate effects of the operation prolonged swinging not only failed to evoke salivation and vomiting, but also exerted no effect on the motility or tonus of the empty stomach.

The depressing effects of motion on the gastric musculature have also been observed in studies made on man. The reports of McDonough (148) and of McDonough and Schneider (151) are of special interest because of the bearing of their results on the question of a possible etiological relationship of gastro-intestinal changes to motion sickness. In roentgenographic studies of the effect of motion (swing tests) on 100 healthy young males it was found that the susceptible or 'sick' group showed a higher incidence of reduced gastric tone or reduced gastric peristalsis or both than the 'immune' group. The differences were statistically highly significant. Pylorospasm was found in some members of each group, either before or after exposure to motion, but its occurrence bore no relation to the development of sickness. There were no significant differences between the two groups as regards the effect of motion on the intestinal progress of a barium meal. Although the frequency of occurrence of a decrease in gastric tone and peristalsis was five times as great in the subjects who became sick as in those who proved to be 'immune,' only one-half of the former showed these changes. McDonough and Schneider point out that their observations did not permit them to predict which individuals would become sick on the swing, and they report their failure to find evidence of disorders or variations from normal in the gastro-intestinal tracts of cadets and crew members who were being eliminated because of repeated and disabling airsickness. These carefully

controlled studies give no support to the view that alterations in the stomach and upper intestine have any significant influence on the genesis of motion sickness.

In a study of the relation of gastric function to nausea in man S. Wolf (230) concluded that this sensation occurs only during gastric relaxation and hypomotility. His report will be analyzed with some care for the reason that it seemed to provide a basis for the use of combinations of atropine and prostigmine in the prevention or treatment of motion sickness (231). He used 3 normal subjects and one with a large gastric fistula. In 5 experiments caloric vestibular stimulation produced nystagmus and vertigo, usually pallor and sweating and sometimes tachycardia. Vomiting never occurred and nausea was induced in only one test. In the 3 tests in which gastric motility was determined, the caloric stimulus was quickly followed by an abolition or marked decrease of stomach contractions. The subject with a gastric fistula was twice exposed to the motion of a swing. In one of these tests no symptoms of sickness developed, but the vigorous gastric contractions decreased sharply after 3 minutes and were not resumed until after cessation of swinging. In the other test the contractions disappeared almost at once, but nearly 20 minutes of gastric quiescence elapsed before the subject experienced some slight nausea. This result contrasts with that obtained in the sole experiment (apparently on a normal individual) in which caloric stimulation produced nausea; the sensation began with and lasted throughout the period of gastric relaxation. In 6 experiments on the subjects who had been given a combination of atropine and prostigmine caloric stimulation did not appreciably affect the gastric contractions and failed to produce nausea, although vertigo was experienced and nystagmus occurred. In view of the fact that caloric stimulation failed to evoke nausea in 4 of 5 tests without these drugs, the conclusion that "gastric relaxation and hypomotility are essential to the occurrence of nausea" seems open to question. A pertinent consideration, which was apparently overlooked in the formulation of this conclusion, is the possibility that the atropine may have prevented nausea by its central action at the same time that the prostigmine maintained gastric contractions by its well-known peripheral action. In their swing tests, McDonough and Schneider (151), encountered several cases of nausea with increased gastric tone and peristalsis.

While it seems well established that various forms of vestibular stimulation, including motion, tend to reduce or abolish the tonus and motility of the stomach, the available experimental evidence does not justify the conclusion that these gastric alterations are in any way causative of the nausea and emesis brought on by exposure to motion.

Studies of circulatory changes induced by motion have been limited to determinations of changes in pulse rate and in systolic and diastolic pressures. The alterations found have been quite small and somewhat inconsistent (31, 47, 57, 94, 138). Cipriani and Morton (57), who followed heart rates by electrocardiographic recordings in 23 men during exposure to the motion of a machine found no constant change; of the 7 who vomited 2 showed slight decreases and 5 showed increases. Hemingway (94), in a study of cardiovascular changes in 489 normal young adults exposed to a swing test, determined that there is a definite tendency for the pulse to slow by 4 or 5 beats a minute in individuals who do not become sick or who develop only mild

symptoms when exposed to swinging. In the severely sick group a small increase in pulse rate was encountered as frequently as a small decrease. Best *et al.* (31) found that swinging tended to decrease pulse rates regardless of the development of cold sweating or pallor. Similarly, systolic blood pressures tended to fall in those who did not become sick or showed only mild symptoms, but rose slightly when vomiting occurred. It may be concluded, especially on the basis of Hemingway's comprehensive survey (94), that susceptibility to motion sickness cannot be related to any specific cardiovascular response to effective motion. The evidence that susceptibility is not associated with a high or low resting blood pressure or pulse rate has been given above.

The facial pallor of motion sickness is a striking and universally recognized characteristic of the disorder. Its onset is a useful sign of approaching sickness, and its persistence after exposure may be used as an objective indication of a slow rate of recovery which, according to McDonough (see 93), is a criterion of susceptibility.

Cold sweating, which is distinguished from thermal sweating by the fact that it has nothing to do with the normal control of body temperature, has been shown by Hemingway (92, 93) to be the most reliable indication of the onset of motion sickness. It occurs when mouth and forehead temperatures stand unchanged or are falling, and it may be profuse even in a cold environment. Hemingway points out that, since in his experiments there was little or no fear, anxiety or apprehension during the onset of sickness, the cold sweating did not depend on emotional or painful stimuli which, as is well known, readily evoke the response.

Several investigators (191, 205, 211) have noted in dogs and cats an increase in frequency and amplitude of respiratory movements when the animals were exposed to rocking, pitching or vertical rectilinear movements. In human subjects hyperventilation sometimes occurs in response to motion; it has been observed during swinging tests and in training flights. Apparently it is seen chiefly in susceptible individuals (198). It may lead to an alkalosis due to carbon dioxide deficit. In extreme cases carpo-pedal spasm and other signs of tetany may develop. This change is to be distinguished from the alkalosis and ketosis which Marrack (145) found in persistent seasickness and which are the results of long-continued vomiting and reduction in food intake. According to Meakins, Morton and McEachern (161) motion sufficient in kind and duration to produce sickness in dogs causes no significant changes in the  $\text{CO}_2$  content,  $\text{O}_2$  content,  $\text{O}_2$  capacity or  $\text{O}_2$  saturation of arterial blood. Cipriani and Morton (57) in a study of 22 human subjects found in most cases that motion produced a slight decrease in respiratory rate and a tendency to sigh and yawn. One subject responded by a rise in rate and developed tetany as a result of the hyperventilation.

Some exploration has been made of the question whether motion produces any chemical changes in the blood. Best, Sellers and Stephenson (32) found a slight decrease in plasma potassium, but no change in the acid-base balance or in the blood acetylcholine esterase in dogs subjected to motion which produced sickness. In similar experiments Babkin and his co-workers (21, 22) were unable to determine any significant or consistent increase in the acetylcholine content of blood. Fields, Meakins and McEachern (68) determined the level of blood sugar, calcium, phos-

phorus, sodium and potassium before and after motion on 13 subjects. Three vomited, 5 showed symptoms without vomiting and 5 were unaffected. The only significant changes (in 11 out of 13 subjects) were: rises in blood sugar of from 2 to 52 mg. per cent, which occurred whether or not the subject became ill; and reductions in blood phosphorus. No other significant changes occurred in the other blood bases. The reasons for the changes in the sugar and phosphorus levels are not clear. It is possible that they resulted from a release of epinephrine, due either to motion or to apprehension. It has been reported that the administration of 1 mg. of adrenalin delays the onset of symptoms in susceptible individuals (234) and that small amounts of pilocarpine or insulin (22 units) given to 5 previously immune subjects resulted in their becoming swing sick (235). It appears that any blood chemical changes induced by effective motion are small and probably wholly secondary to other bodily changes.

Changes in cerebrospinal fluid pressure amounting to 70 to 85 mm. of water have been recorded in a human subject during exposure to the up and down motion of an express elevator (165). There appears to be at hand no evidence which bears on the question whether such rhythmic alterations play any role in the genesis of motion sickness.

The vomiting induced by motion seems to be entirely like that evoked by other stimuli and conditions which bring on emesis. It is the result of activation, through central mechanisms discussed above, of the bulbar vomiting center which is responsible for the orderly combination and sequence of the various components of the act. This mechanism in the medulla discharges impulses over somatic nerves to diaphragm and abdominal muscles the contraction of which supplies the motive power for the emptying of the stomach. At the same time it inhibits respiration. These and other changes in skeletal muscle are preceded and accompanied by alterations in the autonomic innervation of the stomach and intestines which result in decreased tone and motility of the former and generalized contraction of the duodenum (93). Thus, in the act of vomiting, vigorous contractions of skeletal muscle empty the contents of a passive visceral bag. Emesis is a 'somatic,' not a 'visceral' act; the autonomically determined visceral changes accompanying it are in no way essential.

The various bodily changes which characterize motion sickness, the pallor, the cold sweating, the salivation, the gastro-intestinal alterations and the vomiting can scarcely be regarded as adaptive responses to the condition which evokes them. Indeed, from a teleological point of view motion sickness makes no sense. One cannot discern any biological usefulness in these responses to motion; they certainly are not protective. Hemingway (92) has suggested that an explanation is to be sought in evolutionary development. Perhaps it is not too naive to suggest that in the evolution of certain forms the capacity to respond to motion by becoming sick simply developed fortuitously as a by-product of increasing complexity of organization and that natural selection had no opportunity to operate. It is reasonable to suppose that at least man and the dog, probably the most susceptible of animals, were rarely exposed to the adequate stimulus until the former invented or secured various forms of transportation and certain means of amusement.

*Psychological Factors.* The importance of psychic factors in the causation and



control of motion sickness is stressed in a large fraction of the papers which make up the literature of this subject. Many laymen support this etiological concept with the notion that the disorder is indicative of some psychological weakness. Psychiatrically inclined writers have attempted to support it by reference to one or more of the following: *a*) findings of emotional instability and neurotic traits in very susceptible individuals; *b*) claims that apprehension or fear cause or precipitate symptoms of motion sickness; *c*) the precipitation of seasickness or airsickness by disagreeable sights or odors; *d*) the fact that individuals may, through a process of conditioning (in the sense of Pavlov), become sick on motionless vessels or airplanes and, in a few cases, even by looking at or thinking about a moving vehicle or a rough sea; *e*) a supposed correlation between mental or physical activity and a diminution in susceptibility; *f*) stories of sailors and air gunners incapacitated by motion sickness who apparently recovered or at least responded effectively to an urgent call to action; and *g*) reports of the efficacy of non-specific agents such as a lactose placebo in preventing motion sickness.

The view that susceptibility is related to emotional instability and the possession of neurotic traits is based on the psychiatric examination of a few small groups of very susceptible individuals. Schwab (201, 202) examined 115 naval personnel who suffered from chronic seasickness. He divided them into two types. The individuals of Type I were 'constitutionally sick,' presented histories of sickness on all kinds of moving vehicles and devices, and at sea became sick at once and stayed sick so that they were practically useless members of the crew. Type II was composed of men with no history of motion sickness, who at sea carried on with a fair degree of efficiency despite severe sickness. By clinical examination Schwab found the incidence of neurotic traits and neurosis to be much higher in the first than in the second group. He reported that "74 per cent of the Type I individuals show neurotic trends of one sort or another, such as easily upset stomach, a tendency to nausea and vomiting from unpleasant sights, a labile vegetative system so that syncope is common, a history of odd dizzy attacks and so forth." According to Schwab these are vulnerable individuals who develop a definite psychoneurosis as a result of their unpleasant experiences at sea. A somewhat similar conclusion was reached by McDonough and Bond (150). In discussing the management of the airsick aviator they emphasized the fact that symptoms of motion sickness can develop in the absence of any motion. They divided airsick individuals into a 'benign' group, which constituted 90 per cent of the cases, and a 'malignant' group. In the former, sickness began late in flight, occurred only in rough weather and the symptoms were relatively mild. In the latter group sickness occurred even in anticipation of flight and severe symptoms tended to continue after landing. One-half of the 'malignant' group were said to possess such 'neurotic traits' as fear of flying, a history of great fear of heights in childhood, abnormal responses to the sight of blood, inadequate adjustments to family difficulties, and functional gastro-intestinal disorders. Similarly Bond (40), in a study of a group of navigation cadets in the process of elimination for severe airsickness, found a high incidence of "emotional predisposition to neurotic breakdown."

In considering these contentions that there is a specific causal relationship between the psychological make-up of the individual and susceptibility to motion

sickness it is necessary to bear in mind that what has been factually established is that among that small fraction (approximately 10%) of the general population which is highly susceptible some neurotic individuals can be found. A relevant question, which has not been answered, is whether neurotic trends and emotional instability are absent or occur less frequently in those who belong at the other end of the spectrum of susceptibility. Also, there is available no information as to the level of susceptibility among the distinctly neurotic members of the general population. Bond (40) has pointed out and illustrated the fact that the "mere presence of neurotic trends or grossly disturbed family relations does not mean that persons with such histories will be necessarily susceptible to motion."

When this problem was attacked by objective, well controlled psychological methods the results gave little evidence that there is a close relation between common psychosomatic complaints or personality disorders and susceptibility. Birren, Fisher and Stormont (35) studying 277 naval personnel, whose susceptibility was established by several independent criteria, found that a questionnaire, which clearly revealed a significant relation between seasickness and other forms of motion sickness, yielded no evidence of any correlation between susceptibility and any of the following: satisfaction or dissatisfaction with current Navy duty; incidence of headaches, constipation or indigestion; feeling 'weak' at the sight of blood; feeling ill at the sight of disgusting refuse; fainting when giving a blood sample or when receiving an inoculation; apprehension about any aspect of duty at sea. Birren and Fisher (34) found that both their questionnaire and the Cornell Selectee Index (designed to reveal defects of a neuropsychiatric or psychosomatic nature), when applied to a general Navy population or to a selected group of very susceptible men, indicated that 'no large share of the explanation of seasickness should rest on the personality structures of the individual.' In addition, they subjected a group of 48 men, who were so susceptible that they had to be given shore duty and were classified as chronically seasick, to a battery of personality tests. The Bernreuther Personality Inventory indicated that these individuals had lower neurotic tendencies than the standardization groups for this test. The Cornell Index suggested that the seasick group had more psychosomatic complaints than men acceptable, by psychiatric criteria, for military duty, but fewer than is found among psychiatric rejectees. The more subjective tests, the Murray and Rorschach, gave some indication that the group contained more deviates than might be expected in an unselected sample. Birren and Fisher expressed the view that the personality irregularities disclosed were separate entities, associated with chronic seasickness chiefly because there entered into the selection of the men studied the great probability that a commanding officer "is more prone to request the transfer of a man who is a trouble-maker in addition to being highly susceptible to seasickness than of a man suffering from chronic seasickness alone." It was concluded that the tests whose results could be compared with those found in normal populations showed that the group as a whole was "by no means distinguished by deviations in personality structure."

Some writers have expressed the opinion that fear is an important cause of motion sickness. Rubin (197) stated that "the most important causes of airsickness, as seen in a primary air forces flying training detachment, are psychogenic in origin."

While he felt that the group could not be considered emotionally unbalanced, he attributed much of their susceptibility to fear. Green (84, 85) criticized the adequacy of the idea that motion is a primary cause of airsickness and suggested that this disturbance is "a true aeroneurosis, a reaction to fear mediated through the autonomic nervous system and frequently (but not always) precipitated by abnormal motion or altitude of the airplane." Levy (125) emphasized the psychic effect of tenseness in producing airsickness. Witwer (228) agreed with the claims of instructors and the testimony of a certain number of cadets that most airsickness in training is due to fear or nervousness. Zwerling (237) reports that electric shocks applied during rotation in a Spiegel chair increases the incidence of motion sickness. This convinces him that fear, anxiety and personality traits play important roles. Winfield (227) has characterized the recurrence of airsickness under certain conditions as "a psychological escape mechanism." Poppen (189) has expressed the opinion that the cause of air sickness is "practically always an unsatisfactory rationalization of fear," and that in seasickness apprehension is often a contributory factor. On the other hand, one finds references to occasions when the development of situations charged with danger appeared to ameliorate or cure the nausea and vomiting of motion sickness.

The weakness of the observations which have led to the claim that fear, apprehension and tension are important etiological factors is that no account was taken of the influence of visual fixation and head position or of the fact that adaptation to motion occurs under circumstances in which repeated exposures are not attended by any fear, apprehension or tension. Further, those who emphasize the importance of fear and minimize the influence of motion are silent in regard to the fact that the incidence of nausea and vomiting is so very much greater among those who become frightened on boats and planes than among those who experience fear, terror and even panic on solid ground. In his study of the relationship of airsickness to other types of motion sickness Hemingway (95) found that of 344 individuals, who had flown more than 30 hours without airsickness, 11.3 per cent vomited and 16.3 per cent showed pallor, nausea or sweating during a swing test lasting not longer than 20 minutes. It is reasonable to assume that these men experienced more tension, apprehension or fear while flying than during the swinging. Howlett and Brett (110) found that every member of a group of persistently airsick subjects showed symptoms of sickness when subjected to a swing test. They point out that "Amusement was the emotion commonly exhibited before the tests. Apprehension was at no time obvious."

It is often stated that the sight of the vomiting of another individual or exposure to a disagreeable odor induces seasickness. It is true that even ashore such experiences will cause uneasiness and even nausea in a portion of the population. Aboard a moving ship a greater number appear to be sensitive to these influences. While this may be termed a 'psychic' effect, it cannot be regarded as of primary importance in the production of motion sickness. Many of the passengers on a moving vessel are affected to various extents by the motion, that is, they suffer from various degrees of motion sickness, and it seems clear enough that certain sights and odors can serve as stimuli which facilitate the effects of the motion. Similarly, movements which alone do not suffice to produce nausea and vomiting in a given individual may bring on severe sickness if experienced during a time when some other factor is exerting a mildly nauseating effect.

It is true that highly susceptible individuals may become conditioned, especially if their work subjects them to frequent exposures to motion. Thus they may come to show symptoms of motion sickness in the absence of motion. There is also some evidence that similar conditioned responses can be established in experimental animals (26, 191).<sup>3</sup> In our experience, the number of individuals in the general population who do become conditioned is quite small. Adaptation is more frequently encountered. In this connection it may be suggested that the readiness with which a highly susceptible person may develop conditioned responses to odors and sights does not necessarily constitute evidence of a neurotic predisposition.

Physical activity has been recommended by many ship's doctors as a means of controlling seasickness. That any beneficial results obtained by this procedure are due to psychological effects is questionable in view of the fact that moving about may bring into operation one or more specific influences, such as positional or visual factors, which are known to reduce sickness rates. It is generally agreed among sufferers that exposure to fresh air, obtained by going on deck, relieves seasickness. That this is not due to cold air is suggested by Hemingway's demonstration (96) that there is no significant difference in the incidence of swing sickness in cold and hot environments. Again, the contention that it is attributable to some psychological change fails to take into account the fact that going on deck is likely to bring into action ameliorating visual factors. Mental activity has been regarded as an effective means of diverting attention from the malady and so of gaining relief, but the mental activities of aerial navigators and radio operators do not protect them from motion sickness, for these individuals show the highest sickness rates found among members of an air crew (84, 91, 93).

An argument which has very often been advanced in support of the view that psychological factors play a prominent part in the genesis of motion sickness is that a placebo will reduce the incidence. On *a priori* grounds this seems reasonable in the case of individuals familiar with the nauseating and emetic effects of motion. On the other hand, as Smith (206) has pointed out, the administration of placebos to a group of men naive in respect to the potentialities of exposure to motion should, on the same assumption, augment the sickness rate by making the subjects aware of the possibility of becoming sick. Since the impression that a placebo is protective is widespread and has greatly influenced thinking on the subject of the etiology of motion sickness, we shall examine in some detail the available evidence pertinent to this question.

A number of reports of sickness rates in tests on experimental devices have referred to placebo effects. In their early study of swing sickness, Brown, McArdle and Magladery (47) found in a group of subjects (number not given) that "Dummy tablets (lactose) produced no significant difference in the results of swinging except

<sup>3</sup> It should be emphasized that the development of conditioned vomiting as the result of repeated exposure to effective motion is rare among dogs. Although Bard and his collaborators, in an extensive series of observations (26, 27) on susceptible dogs subjected repeatedly to the motion of a swing, encountered a few instances of salivation when the animals were brought into the experimental room or placed on the motionless swing, they never saw conditioned emesis. Furthermore, Noble (see footnote 1) reports that "When animals were swung every 7 or 5 days even though this was continued over a period of years, no case of conditioning was ever encountered." In dogs, as in men, repeated exposure to an effective motion is more apt to result in a decrease than in an increase in susceptibility.

in one individual." Fields, Campbell and Penfield (66) in drug trials on naval ratings, who without any treatment had experienced nausea or had vomited during an initial test on a swing or rocker, gave placebos to 14 men. They state: "Only two (14%) were protected by the treatment and one (7%) partially protected. All three of these had been completely protected by V-1 about a week previously suggesting either a prolonged effect of V-1 or a psychological effect from having been previously protected." The small number of subjects used hardly permits any conclusion regarding protection. In Noble's trials of the effect of drugs on swing sickness (173) there was only one experiment which could possibly yield information about the effect of a placebo. One week after their first test, for which they had been given a capsule of lactose and in which they went the full 30 minutes without vomiting, 24 subjects were swung without any treatment. Three vomited. One of these belonged to a subgroup of 11 who on the first trial had received lactose in pink capsules "at a time when the protective action of pink capsules against seasickness was widely publicized." The other two were among the subgroup of 13 which had been given the sugar in ordinary white capsules. Noble's comment on the result of the second test is: "This would suggest that 12 per cent may have been protected by placebo treatment. On the other hand it may represent the number of individuals whose time of vomiting varies from 25 to 35 mins." Also he concludes that placing the lactose in pink instead of white capsules did not enhance its value as a preventive (see also 183).

In swing trials of various proposed remedies several groups of Canadian investigators (64, 184, 186, 203) used more than 2000 subjects who were graded as susceptible on the basis of the development of severe symptoms during a test which followed the administration of a placebo. Subjects who appeared to be protected on a second placebo trial were removed from the results of the therapeutic trials. Sellers, Parker and Stephenson (203) rejected 67 (28.6%) of 234 subjects because they were either "improved by the psychic effect of the capsule" or "varied from their state at the initial swinging." In a total of 51 tests Parker, Sellers and Stephenson (186) found that the average time on the swing was 29.4 minutes (20 trials) when the placebo trial followed the therapeutic trial and 26.0 minutes (31 trials) when it preceded the testing of the remedy. On the fifth test Fields (64) administered placebos to 46 subjects who had become sick on their first test which was also a placebo trial. The results indicated that 8 were protected and 6 improved (a total of 30.3%). But since the fifth test had followed the trial of an effective remedy by only 3 to 5 days, Fields questioned the validity of ascribing the protection to the placebo. Most of these reports, however, place some emphasis on the supposed protective action of a placebo, but none unqualifiedly asserts that such protection is the only explanation of the results obtained. Doubtless re-testing with a placebo is a necessary check on the susceptibility of the subject during the course of such therapeutic trials. It may serve as a rough correction of the percentage protection observed during the previous trial. But under the conditions of these experiments failure to become sick during the second test of a placebo cannot be taken as evidence of a psychological effect. Indeed the writers of these reports acknowledge the possible operation of two other influences: a prolonged action of a drug given a few days previously, and variations in a given individual's time of vomiting. A third influence is of course the adaptation which

occurs in the majority of susceptible subjects when they are exposed repetitively to the motion of a swing (120, 141), even when successive tests are at weekly intervals (118).

In their recent review of the therapeutic studies sponsored during the War by the Canadian National Research Council, Noble, Sellers and Best (175) state: "In all sea trials definite protection (of the order of 20 to 30%) was afforded by placebo treatment as compared with untreated groups."<sup>4</sup> This declaration is apparently based on results summarized in a subsequent paragraph which refers to tests carried out on troop transports (large passenger liners). These results, originally reported by Parker *et al.* (185), were as follows: On the first day of *trip 1* a '1st sitting' of 220 men received placebos and showed a sickness rate of 10.5 per cent, while only 4.1 per cent of the members of a numerically equal '2nd sitting,' who were given a remedy, became sick. On the second day, with sea conditions the same, neither group received anything and the sickness rates were 17.3 and 15.5 per cent, respectively. Even if it were justifiable to compare the sickness rates (10.5 and 17.3 per cent) of the '1st sitting' on successive days, it is evident that for the numbers used the difference is without statistical significance. In the case of *trip 2*, at the outset of heavy weather on the fourth day, 200 subjects were given a remedy, 160 a placebo and 211 nothing. The sickness rates (incidence of nausea or vomiting) in these three groups were 6.0, 12.5 and 28.4 per cent, respectively. Although the difference in incidence between the placebo and untreated groups is statistically significant, the absence of any information about the method of selection of the groups and their distribution, supervision and duties aboard the large ship raises some doubt that the placebo actually exerted an effect. In another trial aboard a transport the sickness rate was 36.4 per cent in a group of 85 men who were given a placebo and 51.7 per cent in a control group of 87 who received nothing (181). Parker, who reported this work, did not claim that the difference indicates a placebo effect and he was careful to point out the unsatisfactory conditions of the experiment. The subjects were divided into groups not at random but on the basis of their boat stations during emergency drill; the men belonging to the placebo and control groups were not from comparable parts of the ship; there was no supervision of them during the experimental period; and, because they were French Canadians, the symptoms experienced were determined by group questioning through an interpreter.

To the best of our knowledge the facts set forth in the last three paragraphs constitute the entire material on which claims of the efficacy of a placebo may be made. Standing in contradiction to this material are the results of three investigators who examined the problem under more favorable and better controlled conditions. In his study of the effect of hyoscine on airsickness Lillenthal (128) was able to compare the sickness rates which followed the giving of a lactose placebo with those obtained when nothing was given. His subjects were cadet navigators homogeneous as regards age, daily activities and flying experience. Previous to the study they had had 200 or more hours in the air and thus had probably undergone considerable adaptation. The only uncontrolled variable in the flights was the degree of turbulence

<sup>4</sup> The reference given after this statement must be a typographical error, for it is to the analysis by Luykx (132) of the results of sea trials in which there were no untreated groups.

"but the study was extended sufficiently long to make the individual test groups comparable." The control procedure was 531 cadet-flights without treatment during the first two months of observation; it resulted in a sickness rate of 7.5 per cent. This was followed by a period in which, on 200 cadet-flights the incidence fell to 0.5 per cent as a result of giving hyoscine. During the last period, when a placebo indistinguishable from the hyoscine was given, the rate was 6.3 per cent in 239 cadet-flights. The reduction in the sickness rate from 7.5 to 0.5 per cent is impressive, the possibility that it was due solely to chance being less than one in 1000. The difference between 7.5 and 6.3 per cent is without any statistical significance. Smith (207) also was unable to find any effect of a placebo on the incidence of airsickness. His subjects were 274 navigation students selected in the sense that most of them had been airsick 3 or more times when they were given, before each of 5 successive flights, one of the following: remedy A, remedy B, remedy C, a placebo or nothing. The order of these procedures was changed in successive flights and there were 961 individual tests. A placebo was given in 204 tests, nothing in 187. The incidence of vomiting was 41.2 per cent in the former, 42.3 per cent in the latter. On the other hand, actual medication resulted in vomiting rates of 13.5, 19.9 and 21.5 per cent for the 3 remedies tested. In Tyler's 4 experiments (220, 221) involving 563 unselected young men (infantrymen) undergoing shore-to-shore amphibious training in small landing craft (LCVP's) the incidence of seasickness averaged 35 per cent (range, 30-43%) in the 4 untreated groups and 34 per cent (range, 26-46%) in the 4 groups that had been given a placebo and *told it was an effective remedy*. Further, the incidence of severe sickness was 15 per cent (range, 8-29%) in the placebo groups and 13 per cent (range, 9-21%) in the untreated groups. In each of the 4 experiments the members of these groups, together with those of 2 other groups who had received a remedy, were distributed at random in the several identical boats and exposed simultaneously to the same sea conditions. Further, none of the observers and recorders knew the distribution of the men belonging to the 4 categories. The results of these 3 well controlled studies, each differing from the others in the kind of subjects used, make most dubious any assertion that the giving of a placebo with its attendant suggestion can influence the incidence of motion sickness in groups of normal young individuals.

Relevant to a consideration of possible psychological factors in the etiology of motion sickness is the fact, emphasized by Smith (206), that many drugs which possess sedative, hypnotic or anesthetic properties are quite ineffective in preventing motion sickness. While several barbiturates have been shown to depress the emetic response to motion this characteristic is not necessarily related to their hypnotic or anesthetic properties; actually the most effective of these has convulsant rather than hypnotic properties (173). An observation which possibly bears on this question is that in the dog removal of the entire cerebrum in no way diminishes susceptibility to motion sickness, whereas ablation of a small subdivision of the cerebellum abolishes susceptibility (see above).

In summary, it can be stated that the results of relevant, adequately controlled studies do not permit the conclusion that psychological or psychopathological factors are of importance in the etiology of motion sickness. Finally, it may be pointed out that such factors may exert an influence after sickness has developed, for it is well

known that they affect the behavior and the capacities of individuals during the course of any disability.

#### SELECTION TESTS AND PREDICTION OF SUSCEPTIBILITY

During the War a good deal of work was devoted to attempts to develop methods which might serve to predict susceptibility to motion sickness. It became obvious that much time, effort and expense would be saved if it were possible with accuracy to exclude from certain training programs, especially those of the air forces, individuals of high susceptibility. Three types of procedures were investigated: special vestibular function tests, tests on swings and other effective experimental devices, and the taking of individual histories of response to motion by means of questionnaires.

*Vestibular Function Tests.* Early students of this problem attempted to associate susceptibility to motion sickness with abnormal responses to the Bárány chair test. Because nausea and vomiting occasionally occur in rotation tests and because of the evidence that motion sickness is vestibular in origin, the United States Army Air Forces for some time required that all candidates for flying training, who had a history of motion sickness, take a specified Bárány chair test (49, 93). Investigators at the AAF School of Aviation Medicine, Randolph Field, studied the question whether this test could be used as a means of prediction. Campbell and his collaborators (53) applied it to 58 subjects, 30 of whom had definite histories of immunity or susceptibility. They failed to find a good correlation between abnormal responses to rotation and susceptibility, but because of the smallness of the group definite conclusions could not be drawn. McDonough and Thorner (152), in a more extensive study involving over 400 men, were unable to detect any difference between the incidence of swing sickness or airsickness in two groups, one of which qualified and the other failed to qualify in the Bárány chair test. Accordingly the test was abandoned for the purpose of predicting airsickness (97).

Spiegel and his collaborators (216) suggested that their rotating-tilting machine might be used as a means of selection. They found that the weaker method of stimulation (rotation combined with sagittal movements of the head) would reveal highly susceptible individuals.

Caloric tests of vestibular function have also been employed in attempts to pre-determine susceptibility. Goehring and Schwab (83), who tested by irrigating one external auditory canal with 25 cc. of ice water, reported that "subjects who were susceptible showed persistent and marked vertigo, nystagmus, nausea and a degree of falling reaction. Non-susceptible men had no such symptoms nor any transient vertigo or nystagmus." On the other hand, Morton, McNally and Stuart (165, 167) could not find any definite correlation between the vestibular responses of 25 subjects to minimum cold caloric stimulation and their liability to sickness on an effective motion machine. At least two groups of English investigators (47, 86) have reported that there is little correlation between the type of nystagmus obtained in carefully standardized caloric tests and susceptibility to airsickness. The Randolph Field group (152) found that when an evaluation scoring system was used for the responses to both the chair and the caloric tests a 'moderate degree' of correlation was obtained.



*Tests on Experimental Mechanical Devices.* When standard procedures were adopted for swing tests and such important variables as the position of the head, angle and frequency of swinging, vision, etc. were controlled, the results of most investigators have indicated a high degree of correlation between susceptibility to swing sickness and to airsickness. Hemingway (95, 97) obtained the following sickness rates in uniform swing tests: in 438 non-selected, pre-training glider pilot candidates, 28.6 per cent; in 16 cadets hospitalized for airsickness, 100 per cent; in 35 airborne infantrymen who were airsick in a maneuver, 80 per cent; in a group of 73 navigators who were about to be eliminated because of airsickness, 91 per cent. Park (179) found a 67 per cent correlation between swing sickness and airsickness. Brown, McArdle and Magladery (47), using a 15-minute bout of swinging supplemented by 7 minutes of rotatory movements, found that of 15 individuals who had been rated as susceptible to airsickness 10 became sick, while only 5 of 22 inexperienced subjects succumbed. Holling and Trotter (104, 106), however, reported that they were unable to find a good correlation between susceptibility to swing sickness and a record of seasickness. Noble (173) is of the opinion that swing tests are too severe and tend to classify too many as susceptible.

Canadian investigators (63, 165) reported a good correlation between a past history of susceptibility to sickness on ships, aircraft or trains and results obtained on the 'roll-pitch rocker.' Of 26 'immune' subjects only 1 (4%) showed severe symptoms during 30 minutes on the machine while 28 of 41 'susceptibles' (68%) became sick. In 108 men with unknown histories the incidence on the machine was 32 per cent.

In their study of 477 naval officer-candidates Wendt and his co-workers (10) analyzed the relationship of the results obtained on the vertical accelerator to previous history of motion sickness as determined by a questionnaire. The results of this carefully controlled study "showed a reliable and moderately high relation between sickness history and experimentally produced sickness." The over-all rates of sickness on the accelerator were: 45 per cent in susceptibles, 24 per cent in intermediates, and 14 per cent in the group of non-susceptibles. The corresponding 'sickness indices,' determined by giving a double weight to vomiting and single weight to lesser symptoms, were 65, 35 and 21. It was concluded that the sickness evoked by the machine "has factors in common with other forms of motion sickness."

Despite the good correlation reported by a number of workers, it appears that both unnecessary elimination and undesirable retention would occur if selection were based on performance in a swing test. McDonough (149) found that 65.7 per cent of 380 navigator cadets were airsick on one or more occasions; the average was 3 times. Five per cent were eliminated because of airsickness, while the remaining 60.7 per cent were graduated in spite of the fact that they had been airsick on occasion. If susceptibility to swing sickness had been used as a means of prediction 14 of the 19 who were eliminated would have been dropped from the training program, but 130 others of whom 39 never became airsick would have also been eliminated with them. Park (179) found that on the basis of a swing test lasting 30 minutes 12 per cent of a group of candidates would have been rejected unnecessarily and 19 per cent would have been accepted for training and subsequently found unsuitable because of susceptibility to motion sickness. When he reduced the time of swinging to 20 minutes,

the results showed that 7.5 per cent would have been rejected unnecessarily and 20 per cent, later found to be unsuitable, would have been accepted. A further reduction in swinging time to 15 minutes gave essentially the same result. Joeke (120) has suggested that the use of a single swing test is of little help in pre-selection of personnel, since it supplies no information regarding the ability to adapt. He found that of the number who were sick on the first swing test only 1.7 per cent failed completely to adapt and a further 1 per cent showed only slight adaptation. This work and that of Manning (141) strongly suggest that failure to adapt to the motion of a swing might be a reliable criterion for rejection of candidates for duties in which they are exposed to effective motions. Hemingway (93, 97) has recommended that the determination of the ability of the individual to adapt is the most practical selective procedure.

*Use of Case Histories (Questionnaires).* A number of investigators have examined the possibility of predicting susceptibility on the basis of a history of past experiences on various vehicles and certain amusement devices. They have used questionnaires which asked about rides on boats, ships, aircraft, trains, automobiles, buses, elevators, roller coasters, ferris wheels, merry-go-rounds, etc. One of the objections to this procedure is the question of the reliability of the answers of candidates to a questionnaire, particularly when they are aware that their military status or future may depend on their replies. The studies of Birren, Fisher and Stormont (35), of Birren and Fisher (34) and of Wendt and his collaborators (10, 225) indicate that reliance can be placed on answers obtained in properly administered inquiries of this sort and that a questionnaire may be useful as a selection or elimination device.

Hemingway (95, 97) reported a definite correlation between a history of motion sickness and susceptibility to airsickness and swing sickness. But on the basis of his study referred to in the previous section, McDonough (149) points out that if elimination of candidates were determined by histories much the same wasting of adequate personnel would occur as when reliance is put on the results of a swing test. Park (179) emphasizes the fact that if he had used a history as a basis of selection, 10 per cent of the subjects would have been rejected unnecessarily (compare with 12% by the swing test) and 19 per cent, later eliminated from training, would have been accepted.

The weight of evidence indicates a fairly high degree of correlation between susceptibility to airsickness or to the sickness produced by swings or other experimental devices and the susceptibilities revealed by a good questionnaire. Nevertheless, for efficient selection within a large group, the taking of histories, like the giving of a single swing test, probably cannot reveal the thing that it is most essential to know, namely, the ability of most of the susceptible individuals to adapt to motion.

#### THERAPY

Before the recent War no adequately controlled study of the efficacy of any supposed remedial or prophylactic agent or measure was carried out. In the case of seasickness the number of remedies which were advocated approximated the number of interested ship's doctors, ship's officers and highly susceptible passengers. No attempt will be made to mention all the measures that have been tried.

When in 1941 and 1942 it became evident that motion sickness would be a problem of considerable moment in war medicine serious efforts to control it began. Exten-

sive sea-borne and air-borne training operations afforded, actually for the first time, the possibility of carrying out tests on numbers of individuals sufficiently large to give results having statistical reliability. Eventually a fair number of adequate trials were performed, but those who conducted them, both in the air and at sea, were often discouraged by the inconstancy of the two elements. Such students of the problem are well able to appreciate the remark of 3 workers (105) in England: "Chronic sufferers from seasickness may be astonished to learn that on most days throughout the year an obstinate and baffling calm haunts the waters round this island." The development and wide use of effective mechanical devices for the experimental production of motion sickness in men and animals not only advanced knowledge of drug therapy, but served to bring about an appreciation of the influence of such factors as head position, vision and adaptation.

*Psychotherapy.* One or another form of psychotherapy has been recommended by a number of authors (30, 43, 114, 189). A few writers have reported beneficial results which they attributed to suggestion, restoration of confidence and the like, but which, if actually obtained, could have been due to the operation of other influences. For example, Levy (125), who dealt with 52 cases of airsickness among cadets during their first few hours of flying, stated that he reduced the sickness rate from about 10 per cent to approximately 2.8 per cent by instituting a 'psycho-physiological lecture,' in which the students were not only given reassuring information but instructed to throw the head back, change position and fix attention on objects on the ground when they began to feel ill. In addition, instructors were persuaded to "take over and fly straight and level" when a cadet showed the early signs of sickness. Similarly, Green (84), after concluding that airsickness is a reaction to fear, an 'aeroneurosis,' managed the problem of 35 members of a combat bomber crew training unit who had been grounded for sickness by reassuring them and giving them graduated short flights in a type of plane which was 'bumpy,' but generally regarded as 'safe.' Further, a combination of phenobarbital and atropine was administered. As a result of these measures 10 were 'cured' and a good many others showed improvement. Because they ignored such factors as the influence of vision, head position and drug therapy and failed to take into account the fact that adaptation to motion is readily obtained in the absence of apprehension or fear, the conclusion of each of these writers that 'psychotherapy' had an influence cannot be taken seriously.

The absence of any acceptable evidence that psychological or emotional factors are of substantial significance in the etiology of motion sickness (see above) suggests that psychotherapy holds little promise of aiding in the control of this disorder.

*Dietary Measures.* These of course have constituted a favorite subject of many seagoing physicians. Much has been written on the kind of food that should or should not be eaten by the susceptible voyager. There is very little agreement. Some recommend fasting, others only partial starvation. Special diets ranging from meals consisting of nothing more than fruit juices to repasts in which the main dish is beefsteak have had their advocates. One notable recommendation (15) was "soup made of horse-radish and rice, seasoned with red herrings and sardines"—together with small amounts of champagne. As regards the use of alcohol and tobacco, the literature is controversial. Some maintain that these should be strictly avoided; others, more

liberal, encourage the swallowing of straight brandy. While the consumption of considerable quantities of champagne is known to result in some temporary indifference to the symptoms of seasickness, experienced sailors report that disastrous results are likely to attend the practice of going to sea with a 'hangover.'

Seasickness or airsickness will occur in an untreated susceptible individual whether he have a full or an empty stomach, and the available evidence (9, 31, 143, 221) indicates that as long as his diet is not one which would provoke indisposition in a motionless individual on land it makes little difference what he eats or when he eats it.

*Mechanical Measures.* According to Levilly (126) and Irwin (115) Dr. Keraudren in 1812 was the first to suggest the use of abdominal binders. There have been several recent advocates of such supports (43, 54, 61, 101, 177, 189); evidently they have been impressed by the possibility that visceral displacement or shifts of blood in the abdomen are of etiological importance. In view of the fact that there is much evidence to show that such changes (even if they do occur) are of no consequence in the causation of motion sickness, it is not surprising that Aiken, Hoffman and Howlett (4) and Brown, McArdle and Magladery (47), in controlled studies, were unable to find that the use of these devices has any value. Neck cuffs have been recommended on the assumption that cerebral anemia occurs as a result of exposure to motion and is a factor in producing sickness (80), but Fraser (77) was unable to find that the incidence of swing sickness was affected by breathing low concentrations of oxygen. It has been recommended (153) that inhaling deeply when the ship rises and exhaling forcibly on the downward movement is a good prophylactic measure. Over 130 years ago Wollaston (232) advocated the reverse procedure.

At one time or another steamship lines have tried special cabins which, like a ship's compass, were swung on a pivot to prevent them from rolling or pitching. Bessemer (see 17), the inventor of the steel process, was the first to suggest this device. Passengers still became sick, for, as pointed out above, the cabins did not eliminate the vertical motions.

Many types of machines designed to adapt individuals to motion before they go aboard ship or plane have been tried or suggested. Their efficacy appears to be limited by the specificity of the process of adaptation. Gibson, Manning and Cohen (81) found that a regime of swinging for 15 minutes a day during 2 or 3 weeks before and during a period of flying failed to reduce the amount of sickness experienced by subjects in Anson aircraft. There are a number of observations to the effect that acrobats, ballet dancers and athletes are more or less immune to seasickness (43, 139, 178). There are suggestions that physical exercise involving tumbling will reduce the incidence (139). Gibson, Manning and Kilpatrick (82) presented evidence that "a simple 8-week active physical training programme including vestibular exercises" reduced susceptibility to swing sickness. Magladery (135) was unable to find that 2 weeks of physical training produced any lowering of susceptibility to sickness in gliders.

There is general agreement regarding the worth of one mechanical procedure: the assumption of a horizontal position. The reasons for the beneficial effects obtained have been set forth in the section on the effect of position.

*Remedial and Preventive Medication.* A review of the literature, including letters

to the editors of medical journals, from the year 1800 to the present shows that almost every item in the pharmacopeia has been recommended or tried. Some of these have been truly remarkable (14, 102, 134).

The ante bellum efforts to determine whether one or another drug or mixture of drugs is effective have been thus described, possibly too generously, by Noble, Sellers and Best (175): "In general, the investigations have been poorly controlled and are based on too few experiments. These defects make most of the findings of historical interest only." If a trial is to yield reliable results it must meet certain basic requirements. In view of the great variations in individual susceptibility, either the subjects must be ones in which this characteristic has been well established or, if unselected, the subjects must be sufficiently numerous. The reason for the latter precaution has been well stated by Holling, McArdle and Trotter (105) as follows: "If the treated and control groups were sufficiently large it would be justifiable to assume that each contained its fair share of men of every grade of susceptibility, from the immune to the most susceptible, and that the rate of sickness in the control group was therefore a good indication of what the rate of sickness would have been in the treated group if no drug had been given." This procedure is the one which must be used when it is not possible to secure the same individuals for repeated tests. Whether the controls should receive a dummy tablet or not or be subdivided into untreated and placebo groups depends on one's concern about the alleged psychological effect of giving a placebo. It is doubtless much more important to make sure that the observers cannot know which treatment (including a placebo, if used) any subject has received. For the sake of economy of numbers many of the field trials of remedies carried out during the war used only a placebo group for control; it was felt that any medication which did not significantly lower the incidence below that found among those receiving a placebo was without value. In the case of selected subjects of known susceptibility repeated tests must be managed in such a way that adaptation does not confuse the results. Another aspect of the problem of procedure which is important is the criteria of sickness used. There is now general agreement that in tests on human beings nausea and vomiting are the only readily determined symptoms which give dependable evidence of sickness. In animal experiments emesis is probably the only reliable criterion. An excellent discussion of the proper methodology for the determination of the relative value of drugs in the prevention of motion sickness, more particularly airsickness, has been given by P. K. Smith (206).

Little can be said of the curative or remedial effects of any drug, for there have been no adequate tests of the action of pharmacological agents on individuals already motion sick.<sup>5</sup> During the half century which preceded World War II the drugs most often employed to cure seasickness were the bromides, chloral hydrate, glycerol trinitrate, amyl nitrite, sodium nitrate, cocaine, morphine, chlorbutanol, chloroform and ether. These were prescribed individually and in various combinations. Obviously a number of them were given to induce some degree of somnolence until the trip or the bad weather was over. In view of the results of later tests of the effectiveness of belladonna alkaloids as preventives, it is interesting to note that Fisher in 1913 (71) and Bohec in 1930 (39) reported that the administration of atropine to already sick indi-

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<sup>5</sup> But see Appendix.

viduals gave very favorable results. There were also numerous reports of the curative effect of barbiturates (87, 99, 100, 101, 158). Because of failure to carry out adequate control procedures most of these studies yielded results which gave no trustworthy information concerning the therapeutic value of the substances employed.

On the other hand, there are now available the results of a number of statistically reliable studies of the use of drugs as *preventives* of motion sickness. In its military aspects the problem is chiefly a matter of preventing the disorder during the several hours of critical amphibious and air operations. Almost all the adequate trials have been directed toward the discovery of drugs which will significantly reduce the sickness rates which may occur under these conditions. For obvious reasons the oral route of administration has been used in all such studies of pharmacological agents.

The present status of drug therapy in the prevention of motion sickness has been competently reviewed in detail by P. K. Smith for the Army Air Forces (206). Much of the material summarized below has been drawn from his presentation. Attention should also be directed to the review (175) of therapeutic studies which were sponsored by the National Research Council of Canada.

A criterion frequently used to measure the prophylactic efficacy of a drug is "the per cent protected" (206) or "susceptibles protected %" (105). It is a figure which gives an approximation of the number of susceptibles protected by the remedy and is obtained by the formula:

$$\frac{(\% \text{ sick in control group}) - (\% \text{ sick in treated group}) \times 100}{(\% \text{ sick in control group})}$$

While this figure is useful in expressing the results of a given experiment, it must be borne in mind that it is valueless if the difference in the numerator is without statistical significance. Reliable differences should have probability values (*P*) of 0.05 or less. Furthermore, the percentage protected may be misleading in comparing the results of different experiments, since the sickness rate in the control group, which depends on the effectiveness of the motion and the average susceptibility of the members, is a factor which determines the effectiveness of a preventive.

The pharmacological agents most extensively studied during the war were the belladonna alkaloids and central nervous system depressants such as the barbiturates. It was reasonable to place emphasis on these substances, for a considerable literature had accumulated which suggested that they may be effective. It appears that the belladonna alkaloids were first suggested for this purpose anonymously in 1869 (16) and what appears to be a possibly valid test of atropine in the prevention of seasickness was made by Beard (29) in 1880. A number of substances were tested because they appeared to relieve nausea in other disturbances such as radiation sickness and pregnancy, while others were studied for less cogent reasons. Although the urgency of the problem made it necessary to test certain drugs on purely empirical grounds, the selection of substances for final field trial was made, whenever feasible, as carefully as possible. Thus the attempt was made always to try a newly proposed remedy first on susceptible animals or on a limited number of human subjects exposed to the motion of some mechanical device. Then, if it seemed promising, a separate series of tests were conducted to determine whether or not the dosage to be used produced any undesirable side effects. In the case of compounds of unknown or poorly known

pharmacological action it was of course essential to carry out extensive toxicological studies on animals.

1. **BELLADONNA ALKALOIDS.** Before World War II there were available several proprietary remedies which contained these substances, but no adequate tests of their effectiveness had been carried out. Evidence which accumulated during the years 1942 to 1945 definitely established the fact that when administered 0.5 to 4.0 hours before exposure to motion, preparations containing one or more of the naturally occurring belladonna alkaloids are effective in reducing sickness rates encountered on mechanical devices (64, 66, 67, 70, 173, 175, 182, 184, 206, 210), in airplanes (127, 128, 207, 208) and at sea (18, 103, 105, 106, 132, 133, 175, 185, 220, 221).

It is of interest that these drugs, so useful in the case of man, appear to be wholly ineffective in preventing motion sickness in dogs. Babkin and Dworkin (21) gave atropine (0.25 to 0.75 mg.) to 4 susceptible dogs and then subjected them to swinging. Vomiting was neither prevented nor delayed, although salivation was abolished. Holling and Trotter (106) and Noble (172) obtained similar results on giving hyoscine to dogs. In experiments not heretofore reported Bard also found that hyoscine (0.65 to 2.0 mg.) failed to protect a number of susceptible dogs from the emetic effect of a swing. On the other hand, dogs are readily protected by a number of barbiturates and this effect is not related to the anesthetic properties of the compounds (172).

a) *Hyoscine* (L-hyoscine, scopolamine) is probably the most effective of the belladonna alkaloids. In 1942 Holling, McArdle and Trotter (103) carried out the first adequately controlled studies of drugs as preventives of seasickness. Their results, obtained on unselected soldiers subjected in groups of about 70 to the motions of minesweepers or trawlers on trips of 4 to 6 hours duration, were reported in the public literature in 1944 (105). Using as criteria of sickness either nausea or vomiting, they found that of a number of preparations tested hyoscine appeared to be the most generally useful. When 0.6 mg. of the hydrobromide was given only 20 per cent of 218 were sick whereas 46 per cent of the 212 controls (given dummy tablets) were similarly affected. This indicated that 57 per cent ( $47-20/47 \times 100$ ) of those who would have been sick were protected. Suggestive but not conclusive evidence was obtained that 1.2 mg. is even more effective than 0.6 mg. The conclusion that hyoscine is a good prophylactic agent received limited confirmation in sea trials carried out as a joint venture by representatives of the Royal Canadian Navy, the United States Navy and the National Research Councils of Canada and the United States. The results, analyzed by Luykx (132), indicated fairly definitely that hyoscine alone (0.65 mg.) and two other compound preparations containing lesser amounts of the substance were effective in preventing seasickness under conditions of moderate roughness. Later in 1944, in the course of some 60 tests on more than 15,000 unselected troops during ship-to-shore and shore-to-shore amphibious training operations, Tyler (220, 221) showed that several drugs or combinations of drugs are effective preventives. When hyoscine hydrobromide (0.6 mg.) was given alone the ratio of the percentage of those receiving the drug (687 men) who became sick, to the percentage sick in the placebo groups, was 0.34. Here the mean incidence in the controls was moderate, namely, 20 per cent, and the average protection 66 per cent. In one trial carried out when sea conditions produced a sickness rate of 53 per cent in the control (placebo)

group of 118 men the same dose of hyoscine protected approximately 49 per cent of the susceptibles in a treated group of 125.

Hyoscine appears to be as effective, prophylactically, in airsickness as in seasickness. Lilienthal (128), whose method of study has been described above in connection with the alleged effectiveness of a placebo, found that the protection afforded by the administration of 0.6 mg. of hyoscine hydrobromide was 92 per cent on 200 cadet-flights. In his study the incidence of sickness in the control groups was quite low: 6.3 per cent in the placebo group during 239 cadet-flights; 7.5 per cent in the untreated group on 531 cadet-flights. But the medication reduced it to 0.5 per cent. Smith (207) who gave a slightly larger dose (0.75 mg.) obtained a protection rate of 67 per cent. In his well controlled experiment a placebo was given in 204 tests, hyoscine in 222; the sickness rates were, respectively, 41.2 and 13.5 per cent. In comparing the results of these two investigators it should be borne in mind that the subjects used by Lilienthal had had far greater opportunities than those employed by Smith to become adapted to motion before being given a therapeutic test. Lilienthal (128) has reported a number of instances of successful preventive therapy with hyoscine in individual subjects complaining of persistent airsickness. Haslam (89) has described similar experiences with persons whose histories indicated a very high degree of susceptibility to airsickness or seasickness.

Evidence of the effectiveness of hyoscine has been obtained in tests on swings. Smith and Hemingway (210) obtained from 44 to 59 per cent protection with doses of 0.5 to 0.75 mg. Canadian groups (173, 175, 182, 186) found that doses ranging from 0.4 to 0.8 mg. gave protection rates of from 29 to 76 per cent (175). Despite one test in which 1.2 mg. gave only 19 per cent protection (203), it is apparent that in general the size of the dose is a factor in determining the degree of protection. It is also evident that when equal doses and comparable criteria of sickness are used there is an inverse relationship between the degree of protection and the incidence of sickness in the controls.

b) *Atropine*, which is a mixture of equal parts of the optical isomers of hyoscyamine, has received less experimental attention than has hyoscine as a preventive of motion sickness, possibly because in therapeutic doses it lacks the primary depressant action of hyoscine on the central nervous system. In their sea trials Holling, McArdle and Trotter (105) found that of 88 men receiving 1 mg. of the sulfate 23 per cent were nauseated or vomited, whereas 38 per cent of 86 controls (placebo) experienced one or both of these symptoms; the protection of susceptibles was 40 per cent. Quite similar results have been reported by Smith (206) and by Smith and Hemingway (210) in studies of swing sickness, in which the dose was also 1 mg. (given one hour before exposure to motion). In a small group of subjects selected for their susceptibility the protection rate was 42 per cent. In trials carried out on 471 unselected subjects, of whom 365 were controls, this same dose gave an estimated percentage protection of 47 per cent. These relatively limited tests suggest, but do not demonstrate, that atropine is somewhat less effective than hyoscine.

c) *Hyoscyamine*. This alkaloid occurs in two forms, the D and L isomers. The usual commercial product is L-hyoscyamine. As pointed out above, atropine consists of a mixture of equal parts of these isomers. The effectiveness of L-hyoscyamine as a



preventive of motion sickness, when used alone, has been tested by several investigators, but it has been used more often in combination with other belladonna alkaloids, especially hyoscine. Holling, McArdle and Trotter (105) found that L-hyoscyamine, in a dose of 0.96 mg., protected 39 per cent of susceptibles against seasickness during trials in which the protection afforded by hyoscine (0.6 mg.) was 53 per cent. It will be recalled that they obtained essentially the same degree of protection by giving 1.0 mg. of atropine (containing 0.5 mg. of L-hyoscyamine). But L-hyoscyamine and atropine were not used on the same trips; each was tested separately against a placebo and hyoscine (0.6 mg.). The results suggested that they are about equally effective and that neither is as effective as hyoscine. Similarly Canadian studies (173, 175) have indicated that L-hyoscyamine is somewhat less potent than hyoscine in preventing swing sickness. There is general agreement, however, that this isomer is fairly effective (67, 105, 175, 182, 210). As regards D-hyoscyamine the only evidence available is that obtained in a single sea trial involving 34 men each of whom was given 2.0 mg. (105). Only 25 per cent of the estimated susceptibles were protected, whereas on the same trip 0.6 mg. of hyoscine protected 75 per cent of the susceptibles estimated to be present in a group of 35. This result suggests that the effectiveness of atropine is chiefly due to its content of L-hyoscyamine, but obviously the potency of D-hyoscyamine as a preventive has not been sufficiently explored. Smith (206) has remarked that the effect of D-hyoscyamine "may not be negligible since there is insufficient evidence that L-hyoscyamine is twice as effective as atropine, as it should be if the effect of D-hyoscyamine is negligible."

*d) Other atropine-like drugs.* Several of the synthetic substances allied to the belladonna alkaloids have been tested (105, 210). They have given little promise of being useful. Employing small groups of individuals susceptible to swing sickness Smith and Hemingway (210) tested homatropine (12 mg.), benzoyltropine (50 mg.) and benzoyloscine (50 mg.) and found these drugs to be at the most only moderately effective. Their usefulness appeared to be definitely less than that of the naturally occurring alkaloids (hyoscine, atropine and L-hyoscyamine) which were also tested in the same group of experiments. Demerol (100 mg.) was found to be devoid of preventive action and in some subjects it caused nausea before they were swung. Pava-trine (250 mg.) when tested on 20 susceptible individuals gave an estimated protection of 42 per cent, but in a later trial involving a larger number of unselected subjects it failed to have any apparent beneficial action. Smith and Hemingway suggest that the substitution of other groups for the tropic acid of hyoscine, atropine and hyoscyamine greatly reduces the effectiveness of these substances as preventives.

*e) Combinations of belladonna alkaloids.* Although hyoscine (scopolamine) seems to be the most effective of the atropine-like drugs, there is some indication that in doses of from 0.8 to 1.2 mg. this member of the series is more apt to produce certain undesirable side effects, particularly those due to action on the 'higher centers,' than L-hyoscyamine (175). Since it is true that the protective effect of these substances is, within certain limits, a function of dosage, attempts have been made to secure optimal action by mixing relatively small quantities of hyoscine with larger amounts of L-hyoscyamine. A commercial proprietary remedy ('Vasano') consisting of the camphorates of L-hyoscyamine and hyoscine was tested during the War on men sub-

jected to the motions of swings (66, 70, 184), boats (28, 103, 105-107, 133) and gliders (180). The estimated protection of susceptibles varied from practically zero to 72 per cent. It has been indicated that this variation was probably due to considerable differences in the alkaloid content of different batches of the preparation (105, 133). When a definite combination of L-hyoscyamine hydrobromide (0.8 mg.) and hyoscine hydrobromide (0.3 mg.) was used, more consistent results were obtained; the percentage of susceptibles protected against swing sickness varied from 35 to 64 (70, 173, 175, 182, 184, 206). To this evidence one may add that secured when this same mixture was given, together with or shortly before 100 to 200 mg. of niacin. This combination was developed in 1943 at Toronto by the Royal Canadian Navy Research Unit and has generally been referred to as 'the R.C.N. remedy.' It was at first thought (184, 203) that niacin augments the beneficial effects of the alkaloids (presumably by increasing the cerebral blood flow), but later work (173, 182) showed that this substance does not add to the effectiveness of other substances and does not alone have any preventive action. The R.C.N. remedy has consistently given good results in tests on swings (173, 182), at sea (132, 185, 220, 221) and in the air (207). From 47 to 73 per cent of the estimated susceptibles were protected in these various trials.

2. BARBITURATES. Certain members of this series of compounds have been incorporated in proprietary remedies. There are several accounts by ship's doctors (87, 99, 158) indicating that one or another of the commonly used barbiturates is effective in seasickness (153), but these observations were not controlled and in some the doses were great enough to produce a degree of hypnosis that is undesirable, especially from a military point of view. In carefully controlled swing tests little or no protective effect was obtained with barbital (325 mg.) (209), amytal (65 mg.) (173), pentobarbital (100 mg.) (203) or seconal (50 and 100 mg.) (203). In one sea trial phenobarbital (65 mg.) had perhaps a slight effect, but another long-acting barbiturate, 'hexobarbitone,' failed to give the slightest evidence of protective action (105). In all these experiments hyoscine gave definite protection. Nevertheless, it should be recorded that amytal in an amount (130 mg.) that produces scarcely any central depression protected 39 per cent of the estimated susceptibles in a group of 76 (28% sick) when the incidence of seasickness was 46 per cent in a control (placebo) group of 70 (220).

The most extensive investigation of barbiturates as possible preventives is one that led to the emergence of a *thiobarbiturate*, ethyl- $\beta$ -methyl-allyl thiobarbituric acid (V-12), as a moderately effective remedy. This work began in Canada in 1942 and was initiated and chiefly carried on by R. L. Noble (170, 172-174). The basic thought underlying these studies was that it might be possible to find a barbiturate or related compound that would depress the central nervous mechanisms involved in motion sickness and yet would not exert any marked hypnotic or anesthetic effects. Many thiobarbiturates were examined. Toxicity studies on rats, mice and dogs were followed by determinations of the potencies of these compounds as preventives in dogs and human beings (170, 172, 173). In susceptible dogs a large number of barbiturates proved to be very effective (172). Human trials of many of these, however, were precluded by the results of toxicity studies. Others, when tested on human beings, were found either to be ineffective or to exert undesirable side effects (66, 170, 173, 184, 186). The substance designated as V-12 completely protected all susceptible dogs

from swing sickness and did not produce any apparent untoward symptoms (172). The dose required (1.25 to 30 mg/kg.) was directly related to the degree of susceptibility of the animal as previously determined. In the case of human beings V-12 is apparently much less effective, at least when it is given in amounts that produce no undesirable side effects. Noble (173) found in two groups of swing trials that 195 mg. protected 23 and 40 per cent of the susceptibles and in another experiment that 65 per cent were protected by 323 mg. given in a divided dose. The larger amount was slightly more effective than hyoscine (0.65 mg.) or the R.C.N. remedy. Others have not obtained quite such good results with V-12. When Parker (182) gave single doses of 390 mg., only 22 per cent were protected in swing tests. In these same experiments 0.8 mg. of hyoscine protected 73 per cent of the susceptibles. In the sea trials carried out by Tyler (220, 221) 56 per cent of the susceptibles estimated to be present in 1346 subjects were protected by V-12 when the sickness rates in the placebo groups did not exceed 27 per cent and averaged 22 per cent. Three different amounts of the substance were given (260, 325 and 390 mg.), but there was no indication that, within these limits, increasing the dose augmented the degree of protection. In other trials, carried out under various sea conditions, Tyler found that a single dose of 325 mg. gave protection rates of 42, 36 and 30 per cent when the control sickness rates were 19, 28 and 46 per cent, respectively. With the highest rate of sickness among the controls, amytal (130 mg.) was certainly just as useful. These results indicate that V-12 is as effective as hyoscine when the sickness rates in the control groups are below 30 per cent, but that with higher rates hyoscine (alone or in certain combinations) is a better preventive. Noble, however, has obtained evidence that when the daily dose of V-12 (325 mg.) is divided and taken with the morning and evening meals better results may be expected. He states, further, that "continuous treatment starting at least a day before the exposure to motion seems to be advantageous."

3. COMBINATIONS OF BELLADONNA ALKALOIDS AND BARBITURATES. Mixtures of this type were doubtless first introduced with the hope that the two kinds of drugs might act synergistically or supplement each other in their effects on the bodily reactions to motion.

The most extensive tests of a combination of this kind were carried out on a preparation developed before the outbreak of World War II by a medical officer of the United States Army, Lt. Colonel L. L. Barrow. This remedy, known as Motion Sickness Preventive, Army Development Type (MSP) was dispensed in capsules each containing amytal (65 mg.), hyoscine (0.2 mg.) and atropine (0.16 mg.). The usual dose was two capsules. In the case of swing sickness Smith (209), using a small group of 19 men who acted as their own controls, found that only 31 per cent were protected. On the other hand, in air trials he obtained a protection of 52 per cent when he gave this preparation to 176 navigation students (207). The R.C.N. remedy yielded essentially the same protection while hyoscine alone (0.75 mg.) appeared to be a little more effective (67% protected). There is ample evidence that MSP is an effective preventive of seasickness. In the first report concerning it, Barrow, Conn and Williams (28) stated that not one of the 85 men riding in small landing craft who were given MSP became sick. The sea conditions were such, however, that only 10.7 per cent of the controls were sick. A later group of observations, analyzed and reported by

Luykx (132), indicated that 76 per cent of 949 subjects failed to become sick in LST's as a result of taking this mixture. Although the sickness rate in the treated groups averaged 0.8 per cent and that in the control groups (placebo) only 3.3 per cent, the difference appeared to be significant ( $P = 0.01$ ). The much more favorable sea trials which Tyler (220, 221), still later, was able to run off the California coast showed clearly that MSP is quite effective. In this series of studies 2056 men took the mixture 1 to 3 hours before undergoing training operations in small landing craft. Approximately the same number received a placebo and were subjected to the same conditions. In the total of 22 trials the sickness rate in the controls ranged from 5.1 to 53 per cent. At the lowest control rate 92 per cent of the estimated susceptibles were protected, at the highest, 49 per cent. When the sickness rates in the control groups were moderate (e.g., 21 and 28%) the degree of protection was intermediate (67 and 78%, respectively). The average protection was 62 per cent. While Tyler's tests certainly demonstrated that MSP is a good preventive they also showed that hyoscine (0.6 mg.) and the R.C.N. remedy are equally good. They provided no evidence that the addition of amytal enhances the efficacy of belladonna alkaloids. Smith (209) has concluded that the effectiveness of MSP in swing sickness is probably wholly due to its content of hyoscine and atropine. It should be recalled, however, that when the amount of amytal present in the mixture (130 mg.) was given alone a moderate degree of protection resulted (220).

The effectiveness of mixtures of V-12 and one or more belladonna alkaloids has been tested by several workers. In the case of swing sickness Smith (209) found that a combination of V-12 (250 mg.) and hyoscine (0.75 mg.) protected 50 per cent of the susceptibles in a group of 104 subjects, but this degree of protection was no greater than that obtained when 0.75 mg. of the alkaloid was given alone. Parker (182) also concluded that the effectiveness of mixtures of V-12 (290 or 390 mg.), hyoscyamine (0.8 mg.) and hyoscine (0.3 mg.) was no greater than might be expected from their content of belladonna alkaloids. In Tyler's sea trials (220, 221) combinations of V-12 (260 or 325 mg.) with hyoscine (0.6 mg.) or with hyoscyamine (0.8 mg.) and hyoscine (0.3 mg.) were tested on large groups of subjects. The results did not indicate that the addition of V-12 significantly enhanced the protective action of the alkaloids. On the other hand, Noble (173) found that the addition of a relatively small amount of hyoscine (0.4 mg.) to a large dose of V-12 (325 mg.) did not significantly augment the usefulness of the latter as a preventive of swing sickness. When he compared the effects of *a*) 195 mg. of V-12, *b*) 0.4 mg. of hyoscine and *c*) a mixture of the two, he obtained evidence that the combination was more effective than either drug alone. Further, Noble presents data suggesting that when these small doses are used certain individuals are protected by V-12, but not by the alkaloid, and vice versa. This may be an indication that the two kinds of drugs act in different ways, a possibility that is perhaps supported by the fact that hyoscine fails to protect dogs against motion sickness whereas in these animals certain barbiturates are very effective (172).

Although conclusive evidence is lacking that the addition of a barbiturate to an optimal dose of one or more belladonna alkaloids increases the specific action of the latter, there are some reasons for supposing that the use of such mixtures may be desirable (175). The National Research Council of Canada has recommended for use

in the Canadian Armed Services a capsule containing hyoscine (0.1 mg.), hyoscyamine (0.3 mg.) and V-12 (130 gm.). This preparation has been termed the 'Canadian Motion Sickness Remedy—National Research Council Formula' (175). The dose is two capsules taken 2 to 4 hours before exposure to motion and, for continued treatment, an additional capsule every 8 or 12 hours. Certainly this mixture should be as effective as any other that has been tested, and there is evidence that it does not produce any toxic effects when given over a considerable period of time (175, 182).

4. OTHER SUBSTANCES. There have been suggestions that the administration of one or another of the vitamins might decrease susceptibility to motion sickness. Reference has already been made to evidence that niacin has no beneficial action. Noble's results actually suggest that it may have an adverse effect (173). Smith (209) and Noble (173) are in agreement that thiamine is quite ineffective. The use of pyridoxine in the treatment of the nausea and vomiting of irradiation sickness and of pregnancy led to trials of the effect of this member of the vitamin B complex in swing sickness. Given in doses of 100 or 200 mg. it proved to be without benefit (209).

In air or swing trials benzedrine (47, 108, 203, 209), ephedrine (180) and methedrine (105) failed to give any significant degree of protection. When benzedrine (10 mg.) was combined with hyoscine, atropine or hyoscyamine the protection rates were definitely less than those obtained when the same amounts of the belladonna alkaloids were given alone (203). There are two reports, however, of estimated protection rates of 62 and 65 per cent when a mixture of 0.4 mg. of hyoscine, 10 mg. of benzedrine and 100 mg. of niacin was given to men subjected to the motion of swings (184, 203).

The effect of adding prostigmine to one or another belladonna alkaloid has been tested in trials on swings (209) and at sea (220). It was hoped that prostigmine might counteract the undesirable parasympatholytic actions of the atropine-like drugs. Also, there was the claim of S. Wolf (230) that nausea depends on gastric relaxation and that prostigmine may be beneficial because it prevents this visceral change. The experiments of Smith (209) and of Tyler (220) have demonstrated that the protection obtained by these combinations, if any, is small. Further, it is evident that prostigmine decreases the effectiveness of hyoscine (209) and atropine (220) and that a combination of syntropan and prostigmine is quite ineffective (220).

5. PROBLEM OF SIDE EFFECTS. Ideally any drug or combination of drugs employed for the relief of motion sickness should exert its action without producing undesirable side effects. From the point of view of the Armed Services it is most important that the administration of a remedy does not interfere with the ability of the men to carry out their duties.

As regards the belladonna alkaloids it is obvious that in large doses these substances will provoke markedly undesirable effects. These are of two kinds: *a*) a central action, which may lead to mental 'cloudiness,' an excited state or even hallucinations; and *b*) various peripheral effects among which are dryness of mouth and throat, cycloplegia, cardiac acceleration and lessened sweating during exercise or exposure to heat. The question, however, is whether any debilitating side effects develop with appreciable frequency when the atropine-like drugs are given in the small amounts that suffice to prevent motion sickness.

There is abundant evidence that single doses of hyoscine hydrobromide ranging

from 0.5 to 0.75 mg. rarely produce any untoward effect except dry mouth (in about one-third of the cases). Smith and Hemingway (210) found that hyoscine (0.75 mg.), atropine (1.0 mg.) and L-hyoscyamine (1.0 mg.) did not influence pulse rate, blood pressure or the near point of vision, but significantly reduced salivary flow. No significant effects on visual performance were noted in navigation students on long flights after they had received 0.6 to 0.75 mg. of hyoscine (128, 207). Keil (122) showed that oral doses of hyoscine greater than 1.5 mg. produce effects on accommodation which significantly lower visual efficiency in 50 per cent of the individuals tested. This amount of the drug, however, is more than twice the dose effective in preventing motion sickness. More pertinent are the results obtained by Keil after 1.0 mg. had been given: of 15 subjects 3 showed slightly lessened acuity, 3 greater acuity and 9 no change. As regards the effect of hyoscine on marksmanship, the most thorough study is that of Corey and Webster (59). The subjects were 60 U.S. Marines who had been firing for 3 days prior to the beginning of the experiment. They continued to fire during the next 2 (experimental) days, after being divided into 2 groups equal in numbers and in demonstrated ability with the rifle. The members of one group received an initial dose of 0.65 mg. followed by 0.32 mg. every 6 hours thereafter during a period of from 24 to 48 hours; the members of the other group received placebos. The placebo group showed a superiority over the hyoscine group (of about 8% hits) that is statistically significant, but this superiority "was expressed as an increased fire power above the pre-experimental norm, while men receiving scopolamine performed with equal efficiency before and after the administration of the drug." The amounts of hyoscine given in these tests produced no untoward symptoms aside from a feeling of sleepiness, some dryness of the mouth and suppression of lacrymation. In a study involving larger groups Tyler (221, 222a) found that the R.C.N. remedy (0.8 mg. hyoscyamine, 0.3 mg. hyoscine, and 200 mg. niacin) had no effect on the marksmanship of either unselected or highly skilled military personnel. The difference between the results obtained by Tyler and by Corey and Webster may have been due to the fact that the former gave a single dose 2 hours before firing began while the latter investigators administered the alkaloid every 6 hours during the 2 experimental days. It is also possible that the predominance of hyoscyamine over hyoscine in the R.C.N. remedy contributed to the difference in results.

When Holling, McArdle and Trotter (105) raised the dose of hyoscine from 0.6 to 1.2 mg. the only side effect noted was dryness of the mouth; drowsiness and eye symptoms were not encountered. Further, they reported that neither this amount of hyoscine nor 0.96 mg. of L-hyoscyamine had any obvious effect on physical performance, ability to shoot or near vision. On the other hand, Canadian investigators (175) have reported that doses of from 0.8 to 1.2 mg. of hyoscine may produce a change in heart rate, dry mouth, cessation of sweating and, quite commonly, a sensation of 'cloudiness.' They found that with similar doses of L-hyoscyamine the unpleasant mental effects were absent, although some dryness of the mouth and occasional visual disturbances might occur. Accordingly the R.C.N. remedy (a mixture of 0.8 mg. hyoscyamine and 0.3 mg. hyoscine) represented an attempt to keep the amount of hyoscine as low as possible and to augment the therapeutic effect by adding hyoscyamine. When this mixture was administered as an initial dose and half-

quantities given thereafter at 10-hour intervals for 40 hours the only adverse symptom obtained was dryness of the mouth (in 40 to 50 % of the subjects); there were no effects on physical or mental performance. A careful study by Birren, Stormont and Fisher (36), in which a battery of tests covering a variety of sensory and motor tasks were employed, revealed that neither the R.C.N. remedy nor two other effective mixtures containing belladonna alkaloids exerted any significant untoward actions; again, about half experienced dryness of the throat. There is agreement that the diminution in sweating produced by even the larger doses of atropine-like drugs is not sufficient to contraindicate their use in hot climates (105, 175).

It has been pointed out above that in general the barbiturates commonly used in clinical medicine are not effective preventives. Further, it is obvious that even in moderate doses they are apt to produce a degree of hypnosis that is undesirable, especially in the case of military personnel. There is, however, some evidence (220) that amytal, given alone in an amount (130 mg.) that evokes little obvious central depression, is a mildly effective preventive (220). Yet it appears that the addition of this same amount to small effective doses of belladonna alkaloids, as in MSP, does not enhance the prophylactic efficacy of the preparation (see above). The 2 grains of amytal doubtless adds to the tendency of the alkaloids to produce drowsiness. This action is certainly one to be avoided when individuals are required to remain alert for long periods of time, as on long flying missions. On the other hand, under conditions of tension and excitement, e.g., during amphibious or airborne landing operations, the addition of a sedative to a motion sickness preventive may be highly desirable. Indeed it has been demonstrated (222) that barbiturates (amytal or V-12) can be given to men who stay awake for over a hundred hours without significantly affecting their performance or their ability to stay awake. In this connection attention should be directed to a study (221, 222a) which indicates that the amytal content of MSP is probably responsible for the fact that marines who received this medication achieved better scores than their fellows who fired after receiving either the R.C.N. remedy or a placebo.

In his extensive study of the thiobarbiturates Noble paid much attention to the side effects of these substances. He and others have carried out toxicological experiments on animals and have tested on human beings those members of the group which in dogs proved to be at once relatively effective and of low toxicity (170, 175). Here it will suffice to confine attention to V-12 (ethyl- $\beta$ -methyl-allyl thiobarbituric acid) which, as indicated earlier, has proved to be the most useful of these compounds. Noble (175) points out that thiobarbiturates in large doses may cause liver damage and that administration of compounds closely related to V-12 has been followed by the sudden onset of fever and agranulocytosis. Yet human subjects who received 315 mg. of V-12 daily for 30 days showed no symptoms and liver function tests remained normal throughout. The immediate symptom of overdosage is sleepiness. On the other hand, when 315 mg. was given as a single or divided daily dose only a small number of subjects became drowsy; this amount appears to be well tolerated by the majority of individuals. Tyler (221, 222a) has shown that a single dose of 260 mg. has no effect on the rapid fire marksmanship of 'expert' riflemen.

## APPENDIX

'Dramamine' as a Preventive and Curative Agent. Just after this review had been completed there appeared three reports that  $\beta$ -dimethylaminoethyl benzohydryl ether 8-chlorotheophyllinate is effective in both the prevention and cure of motion sickness. This compound, supplied under the trade-name 'Dramamine,' appears to have value in the control of certain allergic manifestations; it possesses antihistaminic properties and has some antispasmodic action.

Gay and Carliner (*Science* 109: 359, 1949; *Bull. Johns Hopkins Hosp.* 84: 470, 1949) were the first to test this substance as a therapeutic agent in motion sickness. Their reason for doing so is interesting because it shows how a purely incidental observation may serve as the basis for provocative studies in an entirely new direction. They write: "Among other patients, the drug was administered to a pregnant woman with urticaria who incidentally had suffered all her life from carsickness. It was possible to control the car sickness of this patient at will. A placebo failed repeatedly, but Dramamine gave complete relief if she took 50 mg. a few minutes before she boarded a streetcar." After some preliminary observations on susceptible individuals they carried out a study on troops during the transatlantic passage of an Army Transport. The results obtained led these observers to believe that this substance is "more uniformly effective in the prophylaxis and treatment of motion sickness than any drug so far employed." Since none of the drugs previously found to be of use in the control of motion sickness appears to be universally effective, one welcomes the introduction of a new and promising agent. But a critical examination of the report by Gay and Carliner does not permit the conclusion that Dramamine is more effective than other preparations in the *prevention* of motion sickness. Even their testimony that it almost always effects a dramatically rapid cure in the violently sick may be subject to some questioning on methodological grounds. We refer below to three aspects of the study which seem pertinent in the determination of its reliability and which bear on the question whether there is at hand any evidence to show that Dramamine is actually superior to other remedies.

1. The subjects were divided into experimental groups on the basis of compartments occupied. This is hardly a sound procedure, for it is clear that even in adjacent parts of a ship men may not be subjected to conditions that are comparable. It is significant that during the first 12 hours the incidence of sickness in subjects, who received neither a placebo nor the drug, was 11.6 per cent in compartment 4-E and 33.3 per cent in compartment 4-F. In the only prophylactic test carried out on the entire trip—it ran during the first 48 hours—123 men in compartment 3-F served as the placebo controls for the 134 in 3-E who received Dramamine. Since the spatial relationship of 3-E to 3-F was the same as that of 4-E to 4-F, it is quite possible that a similar difference, one wholly referable to space, was a significant factor in producing the difference in incidence between the occupants of compartment 3-E (who received the drug) and the men in 3-F (who were given a placebo).

2. It has been demonstrated repeatedly that a rigorous evaluation of a preventive requires that it be tested under conditions in which the sickness rates in the control groups range from moderate to severe. Many preparations give excellent protection as long as the incidence in the controls remains low or moderate (10 to 30%), but with high sickness rates (50% or more) their effectiveness falls off and, in the case of certain barbiturates, may be almost completely lost. In this case the prophylactic trial occurred during the first 48 hours and involved a treated group of 134 men in compartment 3-E and a placebo group of 123 in compartment 3-F. In the former no individual developed nausea or vomiting and 2 complained only of 'dizziness.' In the control group 35 men (28.4%) experienced nausea or vomiting within the first 12 hours and apparently no others developed symptoms during the 2-day period of placebo treatment. This is not a high incidence. If we consider also the sickness rates in the untreated groups of compartments 4-E (11.6% of 129 men) and 4-F (33.3% of 99 men) during the first 12 hours, the overall average is 23.6 per cent. Assuming that all these figures are statistically reliable and counting the 2 cases of dizziness in the treated group as sick the percentage of susceptibles protected by Dramamine was 94 per cent. With such moderate sickness rates in the controls both hyoscine and MSP will on occasion give protection rates as high as 80 per cent. However, as the sickness rates in the controls increase the effectiveness of these remedies falls off. That this is also the case with Dramamine is indicated by the report of Strickland and Hahn (*Science* 109: 359, 1949) on the prevention of airsickness by this drug. Under well controlled conditions in



which 55.6 per cent of those receiving placebos became sick 28.7 per cent of the treated individuals were also sick. Here only 48.4 per cent of the susceptibles were protected. Hyoscine has been shown to give this degree of protection under similar conditions. In view of these facts, and in view of the fact that Dramamine has not yet been tested simultaneously either with hyoscine or any other established preventive, it is premature to claim that this drug is more effective than any other substance in the prophylactic control of motion sickness.

3. The reports of Gay and Carliner concerning the alleviation or total cure of severe cases of seasickness by administration of Dramamine are impressive. In these therapeutic trials, however, two uncontrolled factors may have influenced the results to some extent. Control groups did not continue to receive placebos or remain untreated during the periods of observation of those who received the drug. Many subjects who became sick were alternately placed on Dramamine and a placebo. Therefore it is quite impossible to determine to what extent changes in the effectiveness of the ship's movements contributed to the remission of symptoms. For the same reasons it is impossible to ascertain the influence of adaptation which may be great in any continued exposure to motion. That adaptation occurred in many of the men is indicated by the fact that of the 33 in compartment 4-F who became sick within the first 12 hours and then received a placebo 19 (58%) recovered within 2 days and remained well during the rest of the voyage although the lactose treatment had been discontinued. One naturally wonders how many of those who became sick in compartment 4-E and then received Dramamine throughout the voyage owed their lasting improvement to the process of adaptation. Strong evidence that many subjects experienced adaptation is afforded by the fact that of the 187 men in another experimental group who were quickly relieved of severe symptoms by Dramamine, 143 (75%) failed to become sick again when the drug was discontinued. In this connection it is significant that the weather was roughest during the second half of the voyage. Although it seems likely that the influence of adaptation and changes in motion contributed to the overall apparent success of the treatment, Dramamine certainly effected many rapid and complete cures of severe cases of sickness. In 300 instances of sickness in which the drug was given complete relief was obtained in 288 (96%). Whether this substance is superior in this respect to other substances which have been shown to be effective preventives is a question that remains undetermined, for no comparative results are available.

No studies of side-effects have been reported. It seems likely that the substance would induce a certain degree of drowsiness.

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# ZINC IN THE MAMMALIAN ORGANISM, WITH PARTICULAR REFERENCE TO CARBONIC ANHYDRASE

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ZINC IS AMONG THE ELEMENTS grouped together as 'trace metals', a misleading designation which implies that these substances can be identified qualitatively but cannot be measured quantitatively. It has always been difficult to determine whether the occurrence of these elements in biological material is accidental or is indicative of physiological function. If the latter was established or suspected, it still remained to be determined what the physiological role of the element was.

Lack of progress in this field was due primarily to the slowness of development of chemical and physical techniques accurate for estimation of the minute quantities of the elements concerned. Some methods suggested were inaccurate or invalid, and so gave rise to divergence in the results of investigators working at different times. The serious limitations of earlier methods must be borne clearly in mind in the evaluation of the significance of the findings of their users. Similarly, biological experiments based on deprivation in animals have been most difficult to carry out because of the very minute quantities in which some elements may be active in living organisms. Since occurrence of such elements is wide-spread, the preparation of synthetic diets free of them is a most difficult problem, and almost insoluble in animals of the higher species.

It has become evident in recent years that zinc is important in human physiology, and accordingly findings of observations in animals having a direct bearing on this problem will be reviewed here. Previous studies of the biological activity of zinc have emphasized its toxicological aspects, e.g., metal fume fever. This subject, which has been adequately treated elsewhere (60), will not be discussed.

## ZINC CONTENT OF TISSUES AND BODY FLUIDS

*Tissues.* The zinc content of various tissues has been studied by many workers during the past forty years. Various investigators have attempted to determine the absolute quantities of zinc in plants, invertebrates, and the organs of vertebrates including man as a possible clue to its function (10, 23, 36, 42, 45, 62, 83, 98, 126, 137,

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161, 173, 176). The diversity of methods used by these various investigators attests to their inadequacy. On the basis of recent polarographic work and the use of the sensitive dye diphenylthiocarbazone and related compounds, it has become apparent that the findings of investigators working before their development must be discounted largely as inaccurate from a quantitative viewpoint. Furthermore, comparison between these earlier results is complicated by the fact that units chosen to express concentrations vary markedly. Data are reported per gram or kilogram of wet and dry tissue and since these are not absolute standards of reference comparison is difficult with data expressed in terms of grams of ash. In no instance were the organs perfused to free them of blood present, or corrections made for this source of error. It would be desirable that future work would refer the tissue content of zinc to relatively stable biological constituents, as nitrogen, which could serve as standards of comparison. Arbitrarily choosing those data referring to wet weight, it is obvious that zinc is present in all human and other vertebrate organs in quantities varying from 10 to 200  $\mu\text{g/gm. wet weight}$  (83, 98, 166). Most organs including the pancreas contain between 20 to 30  $\mu\text{g/gm.}$  Liver, voluntary muscle and bone, contain about double this amount while the endocrine organs and lymph nodes contain from 60 to 180  $\mu\text{g.}$  Large quantities of zinc have been found in the corneal epithelium, the iris, retina and lens (98, 161). These data deserve critical re-examination on a larger scale than has heretofore been attempted.

The distribution of  $^{65}\text{Zn}$ , a radioactive isotope of zinc, in the mouse and dog, has been studied (118, 144, 145). Animals were serially killed after injecting the isotope, and following sample preparation, the investigators counted the gamma-rays. The amounts of Zn introduced were negligible as compared with the total amount present in the body. Tissue content of  $^{65}\text{Zn}$  was expressed in terms of percentage of the injected  $^{65}\text{Zn}$  found in each organ. No effort to perfuse the organs was made, and the data are not corrected for blood content.

In both mice and dogs the liver contained the largest fraction of the injected dose early in the experiment. There was a gradual decrease of the activity in both species which was more rapid in the mouse than in the dog. The most rapid uptake and loss during the period of study of 160 hours was observed in the liver, pancreas, kidney and pituitary. Least activity was found in the erythrocytes, brain, skeletal muscle and skin. The activities of spleen, gastro-intestinal tract, adrenals, lungs, lymphnodes, bone, heart and thymus were intermediate. It should be noted that red blood cells and bone were the only tissue components which *accumulated* zinc. Unfortunately, the authors did not measure the total zinc content of organs along with their radioactivity content. Therefore, these data are not significant in regard to over-all zinc metabolism. The ratio  $\text{Zn}^{65}/\text{Zn stable}$  would be a far more meaningful parameter than either measurement alone. While the observations of these authors only extended over 160 hours, it has been shown (165, 166) that the isotope could be detected in dogs, normal humans and one patient with myelogenous leukemia (58) for as long as 8 months, and probably a year. In these studies there was no evidence of selective uptake of  $\text{Zn}^{65}$  in any organs anywhere.

Injection into a pregnant dog demonstrated the passage of isotope across the placenta into all of 7 puppies (166). The total stable zinc content of the tissues of

these animals was similar to that of adults, though the ratio of radioactive to total zinc in 3 of these animals killed at intervals during the first postnatal week showed a re-distribution of the isotope in all organs: there was a decrease in concentration in the extremities, and a rise in concentration in the viscera (166).

**Blood.** The literature contains few references to the quantitative determination of zinc in the blood of normal humans and higher vertebrates (21, 23, 36, 81, 85, 101, 146, 168). A comparison of these results is complicated again because of the different methods used and the different units chosen. Using polarography with an accuracy of 2 to 3 per cent (146) the following mean species differences per gram of erythrocytes have been found:

Man 13 $\mu\text{g}/\text{gm.}$	Rabbit 9 $\mu\text{g}/\text{gm.}$
Rat 10 $\mu\text{g}/\text{gm.}$	Goose 6.5 $\mu\text{g}/\text{gm.}$
Dog 9 $\mu\text{g}/\text{gm.}$	

Recent work utilizing a diphenylthiocarbazone technique (167, 170) accurate for 1  $\mu\text{g.}$  with a standard deviation  $\pm 0.1 \mu\text{g.}$  established human statistical norms in terms of commonly used clinical units as follows:

WHOLE BLOOD 1 cc.	ERYTHROCYTES 1 cc. packed MILLION CELLS	PLASMA 1 cc.	WBC MILLION CELLS
8.8 $\pm$ 2.0 $\mu\text{g.}$	14.4 $\pm$ 2.7 $\mu\text{g.}$	3.0 $\pm$ 1.6 $\mu\text{g.}$	3.2 $\pm$ 1.3 $\times 10^{-3} \mu\text{g.}$
	1.3 $\pm 0.20 \times 10^{-3} \mu\text{g.}$		

The zinc content of the cytoplasm and nuclei of the goose, turtle, frog, and fish—all animals having nucleated erythrocytes—has been examined (81, 117). The investigators found about 3 times as much zinc in the separated nuclei as in the cytoplasm. The findings for plasma and erythrocytes are as follows:

	TOTAL BLOOD Zn	PLASMA mg/kg.	RBC wt	ERYTHROCYTES PLASMA wt.
Goose.....	9.8	2.3	7.5	3.2
Turtle.....	14.2	4.4	9.8	2.3
Frog.....	22.2	8.5	13.7	1.6
Fish.....	7.3	4.6	2.7	0.6

This showed an increase in the erythrocyte/plasma ratio with phylogenetic evolution, probably a consequence of the accumulation of carbonic anhydrase in erythrocytes (81).

There is no variation in zinc levels with age or sex. Three-quarters of the blood zinc was found in erythrocytes, 22 per cent in plasma and 3 per cent in leukocytes (168). Though only 3 per cent of the total blood zinc is contained in leukocytes, cell for cell they contain twenty-five times as much zinc as do erythrocytes. The data available do not disclose the possible differences in the zinc content of the various classes of leukocytes. The absence of uniform distribution in the various components of blood suggests that zinc is a physiological constituent of blood; its variations in concentration follow the mathematical pattern of commonly observed biological distribution phenomena.

The nature of the bond between plasma proteins and zinc is not understood at

present (155). Fraction IV-17 has been shown to bind zinc in proportions of 7.4  $\mu\text{g/gm.}$  of protein at  $\text{pH } 7.8$ ; and 3.8 mg. zinc/ $\mu\text{g.}$  at  $\text{pH } 7.0$  (166, 171). However, it is highly doubtful that this is a physiological transport mechanism.

The discovery of zinc in relatively large amounts in leukocytes is surprising, but the role of zinc in leukocytes is completely unknown and there are no clues as to its possible function. Attempts at radioactive tagging in order to study the leukocytic life span have at best been inconclusive (164). The possible occurrence of exchange of zinc across the white cell membrane contributes to the difficulties of interpretation, and, most important of all, the nature of the compound with which zinc is associated in the leukocyte is unknown at present.

*Sperm.* Mann (105) has investigated the iron, zinc and copper content of ram semen and found the following comparative distribution:

	SPERM mg/100	PLASMA ml. semen
Fe.....	.68	.16
Zn.....	.70	.28
Cu.....	.12	.05

*Excretion and Absorption.* In 1927, Drinker, Fehmel and Marsh (39) reported on the zinc content of normal human urine and feces. The data were obtained with a method of questionable validity, but they seemed to indicate that 1 mg. was excreted in 24 hours in the urine and about 10 mg. in the feces over a similar period. There was a rise in fecal excretion when zinc-rich foods, such as oysters, were consumed. No concomitant changes in urinary zinc excretion occurred. Apparently, the excess zinc was passing through the gastro-intestinal tract without being absorbed.

McCance and Widdowson (108) state that the normal human intake is 10 to 15 mg/day, most of which is excreted in the feces; the urinary output appears to be independent of intake. No actual figures are available which define the minimum daily requirement in man with precision. McCance and Widdowson found it striking that iron and zinc vary together in almost all foodstuffs, i.e., substances having large quantities of one have a similarly high content of the other, and conversely.

By means of intravenous injections they found that most subjects react to an overdose by gastro-intestinal excretion, though individually different physiological reactions occur which may be related to the state of tissue zinc saturation. They found the normal urinary zinc per day to be 300 to 400  $\mu\text{g.}$  while in albuminuria it rose to 2.1 mg/day, a seven-fold increase. This loss was not compensated for by diminished fecal excretion. Similar increases in zinc in nephritic urines had previously been found (48), though there was no correlation with the degree of albuminuria. McCance and Widdowson (108) found 5.1 to 10.3 mg/day in feces, depending upon intake.

*Milk.* The quantities of zinc in human, cow and goat milk were investigated (82). The data were obtained by polarography. It was concluded that colostrum has a high zinc content and that in women the zinc concentration falls to 2 mg/kg. of fresh milk during lactation. Definitive studies on zinc utilization with  $\text{Zn}^{65}$  are indicated and would help to solve many of the questions posed.

## PRODUCTION OF ZINC DEFICIENCY IN ANIMALS

In 1922 an interesting series of experiments was initiated (11) by depriving mice of dietary zinc. The difficulties inherent in designing a diet deficient in such a widely distributed element which would simultaneously supply all other essential nutrients were realized. The authors were aware that their diets probably lacked vitamins, the nature of which was not known at the time. In spite of the obvious experimental defects they observed that the controls receiving zinc lived longer than the zinc-deprived animals. However, both groups died (12). Similar findings were obtained by other authors (70).

The experiments were repeated some ten years later (13, 14) with diets improved by the addition of vitamins. The young were separated from their mothers at 21 days. Their zinc-free diet was admitted to contain more than 50  $\mu\text{g}$ . of zinc/100 gm. of diet, but these data are questionable. The animals on this diet succumbed within 14 to 23 days. They failed to grow, lost weight and died with symptoms of ataxia and posterior column disease. The control animals receiving zinc survived, on the other hand, for 57 to 74 days. The authors concluded that zinc is essential for growth and survival of the normal mouse.

Almost simultaneously, similar results were obtained (162) though these workers concluded that the diets were inadequate in other respects as well. These results were contrary to earlier inconclusive results of other workers, probably as a result of more thorough purification of diet (121).

The Wisconsin School has carried these investigations forward, adopting the rat as an experimental animal. By close attention to preparation of diets these workers were able to obtain normal growth of the control animals. The growth of zinc-deficient rats was only one-third as good as that of normally fed ones. The fur softened and turned from black to gray in 6 to 7 weeks. The zinc-deficient animals required 52 per cent more ration to gain a gram in weight. Two animals which were fed zinc adequately after termination of the experiment returned to normal appearance (154). Further work (66) showed that the zinc-deficient rats received 22  $\mu\text{g}$ /day of zinc. Feeding of 30  $\mu\text{g}$ /day of zinc caused marked improvement and 40  $\mu\text{g}$ /day completely removed zinc deficiency manifestations. The controls received 300  $\mu\text{g}$ /day.

Studies of the carbohydrate metabolism in these zinc-deficient animals showed the glucose tolerance curves to be delayed and irregular; there was no glycosuria, however, and the blood sugar was normal, as was the liver glycogen. These findings suggest the occurrence of delayed absorption of glucose from the gastro-intestinal tract (66). The protein metabolism was disturbed also, as evidenced by a distinct delay in the time required for the blood nitrogen to reach a maximum, and by the lower plasma total protein level in the Zn-deficient rats. The efficiency of food conversion to body tissue was much increased by pituitary transplants (66).

Additional work was concerned with the possible effect of zinc deficiency on enzyme activity as causes of the observed disturbances in carbohydrate and protein metabolism. There was a definite lowering of pancreatic amylase and proteolytic activity in zinc-deficient animals which, however, could not be restored by the addition of zinc *in vitro* or *in vivo*. Similar results were obtained in magnesium deficiency and the change was considered to be the result of general debility (67). Zinc had no effect on crystalline pepsin and trypsin. While no changes in bone phosphatase were

found, intestinal phosphatase activity in zinc-deficient rats was considerably decreased, a finding which was regarded by the authors as a possible explanation for the disturbed amino acid metabolism described. In all the animals zinc content of the soft tissues was normal, but that of the bone was lowered (68). The fact that zinc is part of carbonic anhydrase (see below) led Hove *et al.* (69) to study the state of this enzyme in zinc-deficient animals. Though there was a slight anemia, no striking lowering of the enzyme activity was found.

The uric acid content of zinc-deficient rat's blood was found doubled on all diets tested (182). Carbohydrate, fat, protein or nucleoprotein content of the diet do not influence the course of zinc deficiency but the uric acid blood findings are not reversed until 5 weeks after zinc has been added to the deficient diet. This increased uric acid content seemed to be due to an increased uric acid formation, not lack of destruction since uricase activity was completely normal.

Work by Day and McCollum (33) essentially confirms these findings. These workers found retardation of growth in zinc-deficient rats after 2 to 3 weeks. They noted marked eczema in two cases and alopecia in a few rats. The alkaline phosphatase of the blood was markedly reduced but no changes in the kidneys or bone were found.

Carbonic anhydrase activity per unit of erythrocytes was unchanged. Later work by Day in mice (32, 34) showed that zinc deficiency caused a decrease of liver catalase activity which could not be restored by addition of zinc *in vitro*. Liver esterase and riboflavin in heart, lung, kidney and liver was unchanged. Cadmium did not replace zinc in deficient diets. It was found that zinc was one of the causes of sterility in heifers, which could be remedied by zinc oxide administration (119).

A histological study in zinc-deficient rats (33, 57) revealed hyperkeratinization, thickening of the epidermis, intra- and intercellular edema in the skin and mucous membranes of the esophagus and mouth.

Two balance studies concerning the retention of zinc in pre-school and school children respectively (143, 152) add little to the information on fecal and urinary excretion reported by McCance and Widdowson, and the character of the data presented is such that no general conclusions can be drawn.

The only reports on clinical human zinc deficiency in blood and tissues come from the painstaking work of Eggleton (40-42). In Chinese subjects affected with subacute and chronic beri-beri he found the zinc content of toe- and fingernails and skin reduced to half the normal value. The blood zinc was subnormal compared to the author's normal controls. Similar blood zinc changes occurred in nutritional edema and semi-starvation. On the contrary, the liver and kidney zinc and copper contents were increased in similar cases. No changes were encountered in diabetes mellitus. This work, however, is as yet inconclusive and fragmentary. Zinc is indispensable in mouse and rat physiology, and several human pathological states are accompanied by or may be related in some way to zinc deficiency.

#### ZINC INTOXICATION

No attempt will be made to cover the pharmacological and chemical changes in man or animals given zinc, except to point out that the intravenous administration of 2 mg/kg. of zinc gluconate is tolerated well by both dog and man (166). Four

mg/kg. in the dog produces lassitude, decreased tendon reflexes, bloody enteritis and diarrhea and paresis of the hind legs. The electrocardiographic changes are similar to those observed in potassium intoxication (166).

Doses of 300 mg. of zinc gluconate daily were tolerated without apparent ill effect by a patient with myelogenous leukemia. Doses of 15 mg. intravenously in a dog weighing 15 kg. depressed the leukocyte count temporarily and one dose of 100 mg. in a patient with myelogenous leukemia was followed by a fall in WBC count from 300,000 to 150,000 over a period of five days, after which it rose to the previous level. Further intensive therapy totaling about one gram, failed, however, to affect the disease process permanently (58).

There is a series of observations on the effect of injected or ingested zinc on metabolic processes which deserve attention (37, 72).

In a series of cats fed large doses of zinc, Drinker, Thompson and Marsh (38) observed three which developed fibrosis of the pancreas. These findings were confirmed (137). Following completely negative results (61) in rats which were fed a diet containing 0.25 per cent of zinc it was reported (120) that doses as small as 0.1 mg/day of zinc, fed together with iron, assisted hemoglobin regeneration in the rat. One-half a milligram of zinc, however, retarded regeneration. Sutton and Nelson essentially confirmed these findings (157, 158, 160) and noted that rats fed 0.5 per cent zinc as the carbonate per day showed impaired production of erythrocytes which could be restored by termination of the diet; anemia and retarded growth resulted. The largest dose tolerated apparently was between .5 and 10 per cent of zinc of the total diet per day. On the latter diet animals died after four weeks. Terminally, the authors detected an excess of leukocytes and immature erythrocytes in every case. It should be stated clearly, however, that these quantities of zinc given daily are far above the likely physiological consumption of the element. Similarly, the production of glycosuria (159) by means of high zinc intake by stomach tube is not likely to occur under normal conditions.

An interesting paper confirmed these findings (148). It was shown that rats fed a diet containing one per cent of zinc developed a severe anemia in 3 to 5 weeks, ceased growing, and many died before the sixth week. On a diet containing 0.7 per cent a severe microcytic, hypochromic 'zinc anemia' was produced in four weeks, but the animals lived longer. The additional feeding of copper maintained the hemoglobin at statistically significantly higher levels, and a mixture of iron, copper and cobalt maintained the hemoglobin at or close to normal levels. These elements had no effect on the subnormal growth of the animals fed zinc, but supplements of a liver extract produced a significant growth response. The feeding of zinc brought about a substantial leukocytosis which was almost double of normal when supplementary liver was fed. These findings were of great interest in view of the recent findings that zinc is an integral part of leukocytes and erythrocytes and possibly is related to the maturation of both these cellular elements (168).

#### ZINC IN PATHOLOGICAL CONDITIONS

**Cancer.** Interest in the relation of zinc to cancer developed as a result of the reports (36) concerning the occurrence of zinc in many human and vertebrate organs

and that certain tumors were particularly rich in zinc (28-30, 85). On the basis of all present biochemical and chemical knowledge of the techniques used for preparation and analysis of samples studied, all of this work must be regarded as invalid and of value only insofar as it may have stimulated interest in this field. None of this work will, therefore, be considered further.

Michailowsky reported the experimental production of teratomata in the cock's testis by injection of zinc chloride (114, 115). He noted that this tumor rarely occurs spontaneously in these animals. Periods of high normal or induced sexual activity seemed necessary for the effectiveness of these injections (6). The results were confirmed (4) by transplantation of these tumors, and by identical results with zinc sulphate and copper salts (6, 49, 50-52). It is highly questionable that these data have any physiological significance.

This work prompted Bischoff (16) to investigate the possible carcinogenic role of zinc, which is so frequently injected in man contained in protamin-zinc insulin. Using the Marsch-Buffalo strain of mice, bearing inbred adenocarcinomata of the breast, he found that zinc injections into the breast locally actually inhibited the development of carcinoma as compared with injections of normal saline into controls. The significance of these unconfirmed results is not clear. In two papers published in 1939 and 1940 it was reported (155, 156) that malignant tissues have a very high zinc and very low copper content as measured by means of a polarographic method. Rabbits inoculated with sarcomata showed an increased zinc concentration in the tumor as well as in all other tissues, serum and urine. Clinically, similar findings were encountered in patients.

Recent work on carcinomata and sarcomata of the stomach, colon, breast and prostate do not confirm these findings. Rather, the values were slightly lower than normal though the number of specimens examined was not large enough to lend itself to statistical evaluation. The zinc content of leukemic leukocytes has been found to be depressed to a tenth of the normal value (58). Under successful therapy with x-ray and urethane the zinc content of leukocytes rose to normal levels as the leukocyte count fell. Attempts at influencing the disease process by injection of stable zinc compounds (see above) failed to influence the disease process or raise the zinc level of the leukemic cells.

The lowered zinc content of leukemic leukocytes is of interest when compared with data (22) on mouse epidermis painted with methyl-cholanthrene to induce hyperplasia and carcinogenesis. These workers report the ratio of the metal content to nucleoprotein-bound phosphorus in normal skin, epidermis, and again following carcinogenesis. The copper nucleoprotein-bound phosphorus ratio was lowered to 17 per cent of normal and the zinc nucleoprotein-bound phosphorus ratio was lowered to 32 per cent of normal. Similarly, there was a marked decrease in iron and calcium. Seemingly, a new chemical equilibrium was established. The studies on leukemia were only concerned with one metal variable while the work of Suntzeff and Corruthers shows that under similar circumstances in carcinogenesis a rearrangement of the pattern of the cellular metals takes place.

It seems apparent from these studies that the neoplastic cell may have a metal distribution quite different from the normal. Which is cause and which is effect is



impossible to conjecture upon at this juncture. Certainly, these considerations deserve attention in view of the changes which take place in distribution of enzymes in neoplastic cells.

*Other Pathological Conditions.* A report by Scott and Fisher on the zinc and insulin content of normal and diabetic pancreas has evoked much controversy (55, 56, 137, 138). These authors reported that the mean zinc content of normal pancreas from 14 instances of fatal accidents was 0.14 milligrams of zinc per gram of tissue and the mean insulin value 1.7 IU/gm. of tissue. They stated, on the other hand, that the pancreas in 18 diabetic subjects contained 0.07 mg. of zinc per gram of tissue and 0.1 to 0.5 IU of insulin per gram. The authors concluded that the diabetic pancreas contained a quarter the normal amount of insulin and half the normal amount of zinc and a correlation between the two parameters was implied. A spectrographic technique was used, the details of which are not available, but data on zinc contents appear three to six times too high by comparison with results obtained by more recently developed techniques.

These investigations prompted Eisenbrand and Sienz (45) to examine the subject with a dithizone technique (131, 132). Dry and wet weights as well as the fat-free gland were used as internal reference standards. The non-diabetic mean zinc concentration was 30.6  $\mu\text{g/gm.}$  of fat-free gland. The diabetic mean zinc concentration was 25.3  $\mu\text{g/gm.}$  fat-free gland. These values are compatible with the findings of other authors. There was much variability in the data in the so-called normal and diabetic groups. This apparent difference was caused by some sporadically high findings in the control group which raised its mean value. Unfortunately, the control group was heterogeneous and hardly any cases were included which were really normal. On the basis of statistical analysis the authors concluded that the difference between 'normal' and diabetic pancreas could not be considered significant. They decided that there was no discernible physiological relationship between zinc and insulin, and could not confirm the contention that the pancreas zinc concentration was exceptionally high as compared with other organs, or that diabetic pancreas showed zinc concentrations different from normal pancreas (45). An attempt of Horvai to study the influence of hypophysectomy on the zinc concentration of the pancreas permits no conclusions (65).

It has been found recently that the zinc content of erythrocytes of patients affected with pernicious anemia was markedly elevated before therapy with liver was instituted, out of all proportion to the increase in cell size (163, 164, 169, 172). After institution of therapy there was a continuous fall of the zinc content which reached normal levels in 60 to 70 days, a period of time which has been shown to be the mean life span of the red blood cell in pernicious anemia. These data were, therefore, considered to indicate that the erythrocytes in pernicious anemia were gradually replaced by normal cells formed under the influence of liver therapy. It was concluded that iron and zinc deficiencies probably run parallel and probably occur concomitantly.

There is as yet no explanation for the observed elevation of leukocyte zinc in 'refractory anemias' with leukopenia (169).

## ZINC COMPLEXES

**Hormones.** The crystallization of insulin as a zinc salt (133) led to the belief, now widely held, that zinc is an integral part of the insulin molecule. This belief persists in spite of the fact that nickel, cobalt and cadmium can be used instead of zinc in the crystallization (133). Actually Scott and Fisher never stated that zinc was an integral part of the insulin molecule. They merely reported that the metals and insulin combine in a constant ratio. For zinc the percentage in combination was 0.52, for cadmium 0.77, for cobalt 0.44; these percentages were not changed by re-crystallization (134). Moreover, amorphous insulin is as active physiologically as crystallized insulin, and there is no evidence at present that zinc and insulin must combine *in vivo* to form an active compound. There is far more zinc in pancreas than would be necessary for insulin activation (56).

The quantity of zinc in crystalline insulin has been found to be 0.355 per cent (107) while others found it to vary from 0.33 per cent at pH 5.06 to 0.65 per cent at pH 6.53 when the ionic strength was varied (26, 27). Seemingly, no more than two atoms of zinc have thus far been found to be bound by an insulin molecule. The various mechanisms of the binding of zinc to insulin have been studied, and it was concluded that it may take place either in relation to an  $\text{NH}_2$  or to a  $\text{COOH}$  group (44). If all 29 to 37  $\text{COOH}$  groups known to exist in the molecule were to be saturated with zinc the content of the latter would be between 2.7 and 3.5 per cent, a much higher range than that reported by all workers. However, depending on the number of  $\text{COOH}$  and  $\text{NH}_2$  groups participating in chemical zinc binding different results of the percentage of zinc in insulin may be expected.

Understanding of the interrelation between zinc and insulin has been further complicated by the finding that 0.02 per cent zinc added to insulin as the chloride reduced the immediate activity of the hormone by 40 per cent though the potency of the hormone was not affected if enough time was allowed for it to act. Hypoglycemia in mice and rabbits was delayed, i.e., the blood sugar fell below normal later and stayed there for longer periods, but excessively low values did not occur. The total sugar metabolized was about the same with and without the metal. No such effect was obtained when zinc and insulin were injected separately; precipitation of insulin from a zinc insulin solution with trichloroacetic acid restored its initial properties. Nickel caused the same changes as zinc while cobalt and aluminum had only slight effects on the potency of the hormone (135). The hypoglycemic effect of zinc insulin may be greatly enhanced by the addition of protamin (136). Fazekas and Himwich (53) studied the effect of 0.9 per cent of zinc and aluminum on insulin in cats and dogs and found that such large amounts of metal abolished the effect, while 0.09 per cent of zinc in cats gave results similar to those observed previously by Scott and Fisher. It is interesting to notice the inactivation of penicillin by zinc, the effect of the metal resembling that of penicillinase (46).

The relationship between zinc and activation of hormones needs further clarification in view of the fact that zinc has been said to influence other hormones (15, 47, 107). The marked augmentation of the effect of hypophyseal gonadotrophic extracts as judged by production of estrus and changes in the uterus has been reported (15,

47, 97). Similar effects of copper and zinc on follicle-stimulating hormone and luteinizing hormone in the rat have been noted (54). On the contrary, the addition of zinc to equine gonadotropin decreases its activity (88). The physiological significance of these findings is not clear.

*Porphyryns.* It seems certain that a zinc uroporphyrin exists. Watson *et al.* have found the zinc complex of the Waldenström uroporphyrin (183) to be a constant constituent of the urine and feces in cases of intermittent acute porphyria. Urinary excretion alone seemed to be the rule in the acute, intermittent cases. In fact, the occurrence of the zinc porphyrin complex is characteristic of this type of porphyrinuria while the congenital type usually excretes the porphyrin in a free state (185-188). In these cases a zinc uroporphyrin is found in the liver. A zinc coproporphyrin has also been described (188). Zinc coproporphyrin has been found in cases of lead poisoning (113, 188), and its presence in the urine of acute rheumatic fever has been confirmed (71).

Zinc coproporphyrin has been described in bouillon cultures of *C. diphtheria* (31). The investigators were unable to state whether the zinc porphyrin was present in the bacterium or formed in the solution after disintegration. The relationship of zinc to porphyrins is not understood at present and its physiological significance is unknown.

#### ZINC AND ENZYMES

Reference has been made above to the examination of relationships of enzyme systems to zinc deficiency states and will not here be referred to again. Holmberg's mention of 0.13 per cent of zinc in uricase has received attention. The author's own critical evaluation of the probable metal contamination of his 'pure' preparation by the source material has been overlooked (64). Praetorius' recent work disregarding Holmberg's original conjectures as to the source of the zinc makes it virtually certain that uricase is not a zinc enzyme, thus disproving what Holmberg never claimed (124).

It has been suggested that zinc might be an active part of the kidney phosphatase molecule, but the data afford no evidence in this regard (25).

Zinc, copper, manganese, magnesium and iron have been found in kidney phosphatase (106). The presence of so many elements, none of which accumulated as the enzyme was purified, throws doubt on the significance of the observation. Roche *et al.* thought that they could activate alkaline phosphatase by the addition of zinc sulphate in concentrations of  $1.10^{-8}$  mM. Using three different techniques for estimating phosphatase activity they found that the serum alkaline phosphatase of 97 out of 101 non-cancer bearers (96%) was activated while the enzyme in the serum of 156 out of 185 (85%) was inhibited (125). A recent report disputes these findings (17).

Warburg and Christian believe zymohexase to be a zinc complex; but the quantities of zinc present are so small that they may be impurities (184). Yudkin and Fruton have reported the activation of dehydropeptidase by zinc ions (192).

The only physiological role of zinc which has been conclusively proven to date is related to its presence as an active component of the carbonic anhydrase molecule. Because of the established correlation of enzyme activity with zinc, the properties and behavior of carbonic anhydrase will be given special attention.

That zinc is a constituent of the enzyme carbonic anhydrase was first noted by Keilin and Mann (73, 74). Leiner and Leiner (97) and Hove *et al.* (69) corroborated these findings, and Day and Franklin (35) found zinc in carbonic anhydrase extracted from plants. Keilin and Mann (73, 74) found 0.3 per cent zinc in what appears to have been a highly purified preparation of the enzyme from ox blood. Human carbonic anhydrase has never been successfully crystallized. A dithizone method was used in these analyses. Scott and Fisher (139), using analytical methods of inadequate accuracy (130), recorded the finding of approximately half that amount of zinc. They later confirmed their own results by polarographic methods (140). The discovery of the enzyme seven years previously by Meldrum and Roughton (109, 110) and by Stadie and O'Brien (149, 150) working independently was an outgrowth of the work of earlier physiologists who showed that the reaction  $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$  proceeded at a rate far too slow without catalysis to permit the existence of mammalian life; the reaction  $\text{H}_2\text{CO}_3 \rightleftharpoons \text{HCO}_3^- + \text{H}^+$  is extremely rapid and requires no catalysis. The first of the above reactions is accelerated markedly by carbonic anhydrase. Zinc is an integral part of the molecule of carbonic anhydrase, for removal of the metal by hydrolysis results in inactivation of the enzyme (74); inactivation also is effected by substances which combine with zinc such as cyanide (74, 80, 111, 112, 150, 174), sulphide (74, 80, 111, 150), azide (74, 111) and BAL (189); the findings of van Goor (174) and Leiner (97) in regard to sulphide are discrepant.

Other properties of the enzyme include inhibition by carbon monoxide found by some (111, 112, 174) and disputed by others (80), and inhibition by heavy metals, observed by many (111, 129, 174), and disputed by Leiner and Leiner (97). Certain other inhibitors, widely used in attempts to demonstrate the presence and elucidate the function of carbonic anhydrase are ably discussed by van Goor (181).

The enzyme is a protein whose low molecular weight, first suspected by Meldrum and Roughton from its behavior with ammonium sulphate (111), was later estimated to be approximately 30,000 from studies made in the ultracentrifuge (43, 122) and in the Tiselius apparatus (122, 147). Its isoelectric point is in the neighborhood of 5.6. It apparently contains no SH group (59, 142) although Bradfield felt that the enzyme in plants might (19). The enzyme is reversibly inactivated by oxidizing agents (9, 80) and permanently inactivated by heat. Its activity has been found to be greatly influenced by the concentration of various inorganic and organic substances commonly found in blood and tissues (9, 77, 90-95, 97, 99, 102-104, 141, 142, 178-180). It is felt by some (7-9, 75, 76, 78, 181) that the extreme findings of Leiner in this respect (90-95, 97, 99) require substantiation before acceptance.

*Methods of Measurement.* Methods of estimating carbonic anhydrase are based on measurements of acceleration of the rate of combination of carbon dioxide with or release from a substrate. In this connection it cannot be emphasized too strongly that many substances accelerate the reaction  $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$  (79, 80). Accordingly, it must be borne in mind that slight or moderate acceleration of the reaction by complex mixtures, such as extracts of tissues, does not necessarily indicate the presence of carbonic anhydrase itself; all methods now available measure only activity as represented by non-specific acceleration of a chemical reaction, and not the amount of the enzyme present.

The methods themselves fall into two categories: *a*) There are those which measure the rate of combination of introduced carbon dioxide with a substrate mixture at an alkaline  $pH$ ; this may be followed colorimetrically as first formulated by Brinkman (20) and later modified by others (123, 129) or electrometrically, as proposed by Wilbur and Anderson (190). Disadvantages of these methods arise from the fact that 1) there is no linear relation between enzyme concentration and activity (123, 174); 2) the  $pH$  changes markedly and, therefore, determinations must be made at low temperature, i.e., 15° C. or below, and, 3) indicators used in the colorimetric methods inhibit the enzyme to some extent (24, 80, 190). Clark (24) has recently criticized the electrometric methods. *b*) In the second category is manometric measurement of the rate of evolution of carbon dioxide from a bicarbonate solution upon addition of an acid buffer. The original method of Meldrum and Roughton (111) has several disadvantages and its modification by van Goor (181) involves a complicated apparatus and technique. *a*) One disadvantage of the Meldrum and Roughton method is that it uses an arbitrary unit of activity in spite of the fact that the other early workers, Stadie and O'Brien (150) and Kiese and Hastings (80) calculated activity by means of velocity constants; recently, however, Mitchell *et al.* (116) applied a simple method for calculation of velocity constants to the technique and so supplanted the original arbitrary unit with one of functional significance; Clark (24) has contributed further discussion in this regard. *b*) Another disadvantage of the original method lay in the fact that measurements had to be made at temperatures of between 0° and 15° C., usually 0°. It is well known that the use of low temperatures exaggerates the apparent activity of the enzyme (74, 103, 111, 127). For three years a method has been in use which uses the temperature 38° C. (1); this has been found satisfactory for clinical studies. The method was discussed with Roughton and within a very short time he was able to corroborate the fact that higher temperatures than 15° C. could be used; he also showed that acceleration of carbon dioxide output by the enzyme at 38° C. is only approximately 5 per cent of that obtained when activities of the same preparations are studied at 0° C. (117). Moreover, extreme cold inactivates the enzyme (78, 80, 128). *c*) Another disadvantage of the original method was the violent shaking required; this may inactivate the enzyme (89, 93, 99, 128, 129). However, Leiner (89, 93, 99) and Stadie *et al.* (151) showed that the Warburg apparatus could be used, a finding which was corroborated by Altschule and Lewis (1) and later by Krebs and Roughton (84). *d*) The original method yielded data which showed that there was no linear correlation between enzyme concentration and enzyme activity when blood was used although such a correlation was present when purified enzyme was used, but only in very dilute solution (87, 111). A method, using extrapolation, has been devised for surmounting this difficulty in measuring carbonic anhydrase activity in blood (1). *e*) All manometric methods use substrate and buffer mixtures, and  $pH$  ranges which do not permit of estimation of activity of the enzyme *in vivo*. The recently published work of Roughton and Booth (128) appears to represent the application of elegant refinement in calculation to a method of moderate precision and of low validity for mammalian physiology.

The relation between zinc and carbonic anhydrase in unpurified biological material was first studied by Leiner and Leiner (89). These authors using methods of

doubtful validity found a relation between zinc concentration in various organs and the amount of carbonic anhydrase extractable from the latter. These data are difficult to accept because of inadequacy of the methods used. Later, Leiner (96) and Scott and Mendive (142) stated explicitly that no close parallelism existed between zinc content and enzyme activity. This must certainly be true of tissue extracts for in them zinc is present in many forms. However, in a more recent study of carbonic anhydrase and zinc in human blood methods of high accuracy were used (172). It was shown that a close parallelism exists between carbonic anhydrase activity and zinc content in erythrocytes, normal adult human blood and also in blood in a wide variety of diseases. So close was the correlation between the two that it was suggested that zinc be used as a measure of carbonic anhydrase *content* of erythrocytes, a value that otherwise is not ascertainable. The enzyme is present in large amounts in mammalian blood (1, 2, 5, 73, 86, 87, 100, 111, 174-177); it is all within erythrocytes, for no activity is found in leukocytes or in plasma (1, 181); Lambie reported a very small amount of activity in human plasma (87) and Booth found a little in pig serum (18). The best evidence indicates that red cell carbonic anhydrase content of erythrocytes, judging from their zinc content is probably one per cent of the hemoglobin content. The amount of carbonic anhydrase in dog blood is somewhat less, judging from the smaller amount of zinc found in the red blood cells of that species.

The blood of mammalian embryos or newborn exhibits low carbonic anhydrase activity, as was first noted by Meldrum and Roughton (111), van Goor (174) and Lambie (87) and later studied extensively in infants by Stevenson (153); the blood of premature infants has even less activity. The methods used by all of these authors exaggerated the deficiency of the enzyme in the blood; recent studies (1) show a lesser degree of lowering of activity in the blood of newborn infants so that the carbonic anhydrase activity per ml. of blood, enhanced somewhat by the polycythemia common at this stage, is only about 50 per cent less than that of the normal adult, with the blood of premature infants at a somewhat lower level (3). Studies on the zinc content of blood in newborn infants are not available.

In addition to the fragmentary studies of Wille (191) and of Hodgson (63) in anemia, there is a considerable body of data published by Lambie (86, 87) who used the original Meldrum-Roughton technique. Studies on bleeding in mammals have also been reported (87, 178). More recently, studies by a better method corroborated Lambie's (86, 87) conclusions as to the parallel decrease of carbonic anhydrase activity and blood hematocrit cell percentage and hemoglobin concentrations in patients with hemorrhage (100, 172); in addition, it was shown that the blood of patients with anemia associated with nutritional iron deficiency, uremia and infection exhibit similar findings. In pernicious anemia, on the other hand, the blood carbonic anhydrase activity is in or near the normal range in spite of very low hematocrit cell percentages and hemoglobin concentrations (100, 172); this phenomenon is not due solely to large cell size, for the mean corpuscular carbonic anhydrase activity is increased several times more than the increased cell size and elevated mean corpuscular hemoglobin values. In patients with anemia associated with liver disease or with leukemia the carbonic anhydrase may be reduced in proportion to the hematocrit cell percentages and hemoglobin concentrations, or may be disproportionately high,

though not necessarily as much as in pernicious anemia. The statement by Lambie (87) that hemolytic anemias are associated with disproportionately high blood carbonic anhydrase activity is not borne out by his own data, for only one of his eight cases showed this phenomenon; this disproportion was not found in a more recent study either (100). The significance of change, or lack of change in carbonic anhydrase activity in various types of anemia cannot be stated but it is of interest that in pernicious anemia exertional dyspnea is absent or notably mild in spite of low hemoglobin concentrations. At any rate, it is worthy of note that whatever the level of carbonic anhydrase activity in the blood, the concentration of zinc in the erythrocytes is at all times proportional to the activity of the enzyme in anemia (172).

In patients with polycythemia vera, the blood carbonic anhydrase activity is increased in proportion to the hematocrit cell percentage and the hemoglobin concentration (100); a similar situation obtains in patients with polycythemia secondary to chronic pulmonary disease (2). Here again a close parallelism between enzyme activity and erythrocyte zinc concentration is found (172).

Discussion of the carbonic anhydrase content of tissues is beyond the scope of this review; this material has been covered thoroughly in van Goor's excellent review (181). It should be re-emphasized, however, that since zinc exists in forms other than or in addition to carbonic anhydrase in tissues, the use of zinc levels for estimating enzyme content or supporting measurements of enzyme activity is not valid in biological material other than blood. The confused state of the literature regarding the presence and function of carbonic anhydrase in tissues, ably delineated by van Goor (181) is understandable; precise knowledge regarding the enzyme must wait on the isolation and intensive study of the enzyme, the formulation of better methods for estimating activity, and the development of specific chemical methods for measuring the amounts of enzymes present in various tissues.

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# THIAMINASE, THE CHASTEK-PARALYSIS FACTOR

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THE INCLUSION of raw fish in diets fed to foxes on fox ranches has resulted in severe outbreaks of a paralyzing disease which, left untreated, is fatal. R. G. Green first noted this condition in 1932 on the fox ranch of J. S. Chastek of Glencoe, Minnesota. Four years later he published a description of the disease which he called Chastek paralysis (19). Ultimately it was found that Chastek paralysis is due to a thiamine deficiency induced by a heat-labile factor in certain species of fishes which renders the normal dietary thiamine unavailable to the fox. Shortly the Chastek-paralysis factor was shown to be an enzyme which splits thiamine into its pyrimidine and thiazole components, and thus persisted the name thiaminase (56) although this name had been suggested earlier by Bonner and Buchman (8) for an enzyme in pea roots capable of synthesizing thiamine. Three reviews (40a, 41, 1) have appeared concerning the nutritional aspects of thiaminase. Considerable work has been done, however, on the nature and distribution of this enzyme, as well as on the deficiency it produces. This review proposes to cover most of the literature available at present.

## CHASTEK PARALYSIS

*Symptoms.* In the period from 1932 to 1936 Green studied 4 acute outbreaks of a hitherto undescribed disease of foxes. About one month after the introduction of raw fish to the diet of foxes on the ranch of J. S. Chastek, Glencoe, Minnesota, 34 per cent of these animals rapidly developed a spastic paralysis which resulted in death in 1 to 4 days after the onset of symptoms. The fish employed were frozen immediately after catching and thawed only at the time of feeding, so that no spoilage could have occurred. In the very beginning it was shown that Chastek paralysis was neither food poisoning nor was it transmissible from fox to fox as an epizootic disease. These occurrences of Chastek paralysis, moreover, were on widely separated ranches, all of which fed fresh fish. In all cases the disease stopped once the fish was removed from the diet (19).

Once paralysis set in, the course of the disease was rapid and the symptoms became more characteristic (19, 28). Several weeks after the feeding of fresh fish was begun, the foxes began to refuse food. In the interval, however, no harmful effects were evident from the change in diet. The anorexia increased during the following week until some of the foxes were leaving all of their ration. Stiffness of gait, the first sign of actual disease, occurred after a week or two of anorexia. Within 12 to 36 hours these foxes showed extensive spastic paralysis so that they could not rise,

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or stand if they were placed on their feet. The animal displayed a general, abnormal sensitivity to pain and remained conscious although it was almost completely paralyzed. Convulsions often occurred, and the animal usually died within 12 hours after total paralysis of the limbs had set in.

*Etiology.* Green (20) announced that Chastek paralysis was due to a vitamin B deficiency caused by fresh whole fish when it makes up 10 per cent or more of the foxes' diet. Below is a diet which produced *no* outbreak of Chastek paralysis in January 1935 (21).

Cereal . . . . .	10.0%	Fresh fish . . . . .	7.0%
Fresh vegetables . . . . .	10.0	Cod liver oil . . . . .	0.1
Liver . . . . .	8.0	Dry milk . . . . .	2.0
Horse meat . . . . .	50.0	Water . . . . .	12.9

In January of the following year, however, when the diet was modified to include 18.0 per cent raw fish, 2.0 per cent eggs and 2.0 per cent liver, a severe outbreak of Chastek paralysis occurred. At that time the vitamin B deficiency was attributed to the increased demand for the vitamin made on the fox by the metabolism of the fish oils together with the comparatively poor source of vitamin B that fish provides. In the above disease-producing diet the amount of liver and eggs fed was considered not enough to prevent the avitaminosis.

An inherent objection to this hypothesis was that cooked fish could be fed in large amounts with impunity. Since Chastek paralysis was a neurological manifestation it was assumed at first that thiamine was the B-factor involved. Cooking is very likely to decrease, rather than increase, the thiamine content of the fish. Lunde (38) showed that the food items in diets which produced Chastek paralysis contained essentially the same amount of thiamine as diets which produced no symptoms of pathology. Coombes (10) claimed that Chastek paralysis was not caused by a thiamine deficiency because he was able neither to produce the disease in animals fed a thiamine-deficient diet, nor to cure the fish-diet disease by feeding thiamine in large amounts. He attributed the paralysis to specific heat labile toxins occurring in fresh or partially decayed fish. Investigators in Norway, however, were able to produce a condition, the symptoms of which very much resembled Chastek paralysis, by feeding foxes a diet deficient in thiamine (16, 36). Thiamine injected into these animals resulted in the disappearance of paralysis. What appears to have been a typical outbreak of Chastek paralysis in Sweden was checked by administering thiamine to the sick animals and reinforcing the diet with dried yeast (9). Although the presence of fish in the diet was noted, the occurrence of the thiamine deficiency was not linked with the feeding of fish.

It soon became apparent that the thiamine deficiency resulting from feeding raw fish occurred, not because the thiamine content of the diet was too low, but rather because the fish in some way was making the dietary thiamine unavailable to the fox.

*Pathology.* In Chastek paralysis the diagnostic lesions at necropsy are found in the brain when examined microscopically. These lesions were noted in the early studies (19, 21) and were described later in much greater detail (17). Characteristic changes, however, are often found in other organs.

In gross examination the appearance of the heart may suggest Chastek paralysis. It shows massive degeneration with cloudy swelling, in some cases so severe, that the authors use the term 'cooked' to describe it. In about one-half of the animals hemorrhages are present. Microscopic examination reveals large nucleoli and focal necrosis in myocardial fibers, proliferation of connective tissue arising from the vascular tree, and hemorrhages. This combination of changes, although one or more is frequently absent, is indicative of Chastek paralysis.

The liver usually shows degeneration but its gross appearance is quite variable, at times fatty, or of a dark mahogany color. Most of the viscera other than the heart and liver do not show any effects of Chastek paralysis.

Most significant and diagnostic, however, are the lesions which occur in the brain and spinal cord of animals with this disease. Sections of the brain and spinal cord reveal small spot-like hemorrhages which can usually be seen grossly. Microscopic examination reveals that typically the lesions are bilaterally symmetrical and do not extend into the white substance. The changes appear to be vascular; small blood vessels have undergone irregular dilatation and vast proliferation of their endothelium. The lesions involve only certain of the paraventricular nuclei and also are commonly found in the inferior olive and in the cortex of the splenial gyrus. Despite the fact that these changes are vascular they are never encountered in the white substance.

The brain lesions of Wernicke's disease due to chronic alcoholism in man have been shown by Alexander (3) to be identical with those induced experimentally in pigeons by feeding a thiamine-deficient diet with an ample supply of the other vitamins. Additional support for the theory that Chastek paralysis is caused by a thiamine deficiency was furnished by the fact that the distribution and histological appearance of the lesions in this disease were identical with those found by Alexander in his study of Wernicke's disease (27, 4).

*Nursing Fox Pups.* The pathological findings described above are not present in nursing fox pups which die from Chastek paralysis. In these, the disease takes an acute form; Green (22) has described outbreaks of Chastek paralysis in fox pups in which one-third of the nursing pups on a ranch have succumbed in a period of 3 days. The severity of the disease in pups has been attributed to the absence of thiamine in the mother's milk, since lactating females with Chastek paralysis would tend to conserve thiamine for their own use (28). It has been shown that lactating animals with sub-acute vitamin deficiencies induce acute deficiencies in their young, retaining the vitamin for themselves (42). Thiamine analyses, however, have not been performed on the milk of lactating foxes with Chastek paralysis. The absence of the neurological pathology in the fox pups has been explained on the possibility that Chastek paralysis in its acute form does not give a chance for the vascular changes to occur before it interferes with a vital function. Under these circumstances it appears that the vascular changes may in themselves not be responsible for the death of the animal. Treatment of the affected fox puppies with thiamine effected a rapid and complete cure.

*Therapy.* By the end of 1940 the cumulative evidence pointed to Chastek paralysis as definitely a thiamine deficiency (27). Possible seasonal occurrence of the

disease had been explained by the fox farmers' habit of feeding raw fish during the winter months when the foxes' sources of protein become restricted (23). Shortly after crystalline thiamine chloride hydrochloride was available, Green, Carlson and Evans (25) showed that a diet with 20 per cent raw carp which produced Chastek paralysis in controls would not produce the disease at all when it was supplemented by 25 mg. thiamine per day. Moreover, cures of Chastek paralysis were effected by the subcutaneous injection of 20 mg. thiamine although, as was admitted by the authors, the supply of foxes with the disease was too limited to allow the simultaneous use of untreated controls.

The objections by Coombes (10) to the theory that the etiological factor was a lack of thiamine were withdrawn (11). Despite earlier unsuccessful attempts by Coombes (10), Hodson and Smith (30) were able to produce the clinical symptoms of Chastek paralysis in a group of foxes fed a diet deficient in thiamine but containing no raw fish. Brain lesions typical of Chastek paralysis were not, however, found in the only 2 foxes of this group subjected to post-mortem examination. Simultaneous cases of Chastek paralysis for comparison of behavior were obtained by including raw smelt in the otherwise adequate diet of a second group of foxes. Only thiamine, and not riboflavin, pyridoxine or calcium pantothenate, would cure both the deficiency syndrome and the Chastek paralysis. Previously it had been shown that nicotinic acid was not therapeutic (25).

Three possibilities of how fish are able to cause thiamine deficiency presented themselves: "First, fish may somehow increase the animals' need for vitamin B<sub>1</sub> by a metabolic effect; second, fish may prevent absorption of the vitamin; and third, fish may bring about an inactivation or destruction of the vitamin in the food" (25).

#### INACTIVATION OF VITAMIN B<sub>1</sub>

Shortly after the work of Green, Carlson and Evans (25) proving that Chastek paralysis is a manifestation of B<sub>1</sub> avitaminosis many workers took up the investigation to find out how the raw fish rendered the thiamine unavailable. Spitzer *et al.* (55) showed that typical polyneuritis can be made to occur in chicks by feeding 25 per cent whole raw carp in an otherwise satisfactory basal ration. Especially significant are the results of their *in vitro* experiments in which they incubated raw or cooked carp entrails with thiamine chloride hydrochloride and then attempted to recover the added vitamin B<sub>1</sub> by means of the thiochrome method. In this way they showed that the destruction of the thiamine occurs within the food mixture itself and that the inactivation of the vitamin was proportional, more or less, to the length of time it was in contact with the raw carp entrails. In addition, they confirmed the heat lability of the factor and indicated that the raw entrails were more potent than the whole carp in producing chick polyneuritis.

At approximately the same time, Green (24) demonstrated that raw fish could be fed with impunity provided the vitamin B<sub>1</sub> of the diet did not come in contact with it in the diet mixture. The fish and a balanced diet were fed on alternate days. Carp fillets were shown by Green, Carlson and Evans (26) not to produce Chastek paralysis. On the other hand, the disease was readily produced by feeding the external structures (scales, fins, skeletons and heads) or the viscera of carp in the diet. The feeding of 10 mg. thiamine per day was sufficient to prevent Chastek paralysis.

Woolley (59) was able to obtain extracts of high activity to inactivate thiamine *in vitro* by extracting fresh carp tissue with 10 per cent sodium chloride solution. Aqueous extracts of carp, however, had only 25 per cent of the activity of whole carp suspensions. These, like the earlier investigations (55, 26), pointed to the Chastek paralysis factor as being either an enzyme which destroyed thiamine or a protein which combined with whole thiamine to render it unavailable; an analogous situation has been found in the combination of avidin with biotin (14). Additional light was thrown on this by Deutsch and Ott (13) who demonstrated that the thiamine of non-viable yeast is destroyed by keeping it in contact with raw smelt and that the thiamine so destroyed cannot be recovered by mild acid hydrolysis, a treatment which releases biotin from the avidin-biotin complex. Yet, like avidin, the Chastek-paralysis factor was found unable to penetrate the living yeast cell, for the thiamine

TABLE 1.<sup>1</sup> DISTRIBUTION OF THIAMINE-DESTROYING PRINCIPLE IN CARP TISSUES

TISSUE	WEIGHT	ACTIVITY UNITS	TISSUE	WEIGHT	ACTIVITY UNITS
	gm.	per gm.		gm.	per gm.
Spleen	7.8	25.00	Kidney	17.8	1.34
	2.5	6.00	Blood		1.23
	8.2	21.6			0.80
Liver and pancreas	95.1	2.5	Heart	4.1	0.33
	53.9	1.90	Testes	39 <sup>2</sup>	0.25
	34.7	1.48	Brain	2.3	0.21
Gastrointestine	44.4	2.5	Mucous	21.2	0.04
	31.3	8.72		3.7	0.14
	23.7	3.54	Gall bladder and bile	8.4	0.11
Gills	52.1	2.5	Eyes	7.8	0.06
	34.0	4.34	Ovaries	9.3	0.62
	15.2	2.16	Swim bladder		0
			Muscle		0

<sup>1</sup> SEALOCK, LIVERMORE AND EVANS (52).

content of viable yeast was not lowered significantly when the yeast was mixed with raw smelt.

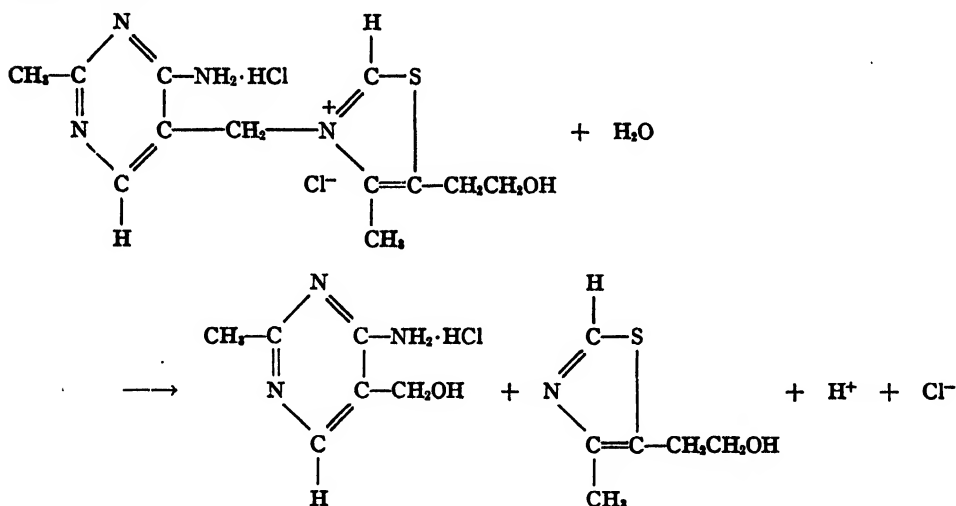
The properties of the *in vitro* inactivation of thiamine were studied by Sealock, Livermore and Evans (52) in some detail. Using Woolley's technique for extracting the factor which destroyed thiamine, they assayed various organs and structures of the carp. Their results are summarized in table 1 from which it can be seen that the greatest activity resides in the spleen of the carp and that the muscle of the fish is inactive. One unit of activity was defined as that amount of activity which at pH 7.4, 37.5° C., at a thiamine concentration of 0.5 micromoles per ml., will cause the disappearance of 1 micromole of thiamine. These authors prepared a stable desiccated powder from acetone-extracted minced carp viscera from which they were able to extract the thiamine-inactivating principle with 10 per cent NaCl solution. With this preparation they found that the maximum disappearance of thiamine occurred at pH 9.1, with suitable corrections for the thiamine lost by alkaline hydrolysis. The activity of the preparation was greatest at 60° C.; raising the tempera-



ture of the extract to 100° for 30 minutes completely inactivated the principle. The disappearance of the thiamine was approximately proportional to the amount of the extract used and fitted more or less the kinetics of a first-order reaction. These properties were advanced by the authors as all evidence in favor of the *enzymatic* destruction of thiamine.

#### ENZYMATIC DESTRUCTION OF THIAMINE

*Cleavage of Thiamine.* Although the kinetic studies of Sealock *et al.* (52) suggested that the inactivation of thiamine by carp tissue was an enzymatic reaction, the final proof of this hypothesis was achieved by Krampitz and Woolley (35) who isolated the products, 4-methyl-5-hydroxyethylthiazole and 2-methyl-4-amino-5-hydroxymethylpyrimidine, of the thiamine cleavage from a thiamine-free mixture of carp tissue incubated with thiamine. The reaction, therefore, was a hydrolytic cleavage of the thiamine molecule between the pyrimidine and thiazole rings as shown below.



It should be noted that this hydrolytic cleavage sets free a proton from the water molecule; the tertiary nitrogen of the thiazole moiety will, in acid solution, form a salt with this proton in covalent linkage, the nitrogen becoming pentavalent. The release of this proton, however, would serve to lower the *pH* as the reaction progressed.

Krampitz and Woolley (35) have suggested that the destruction of thiamine involves more than one step. *Mucor ramannianus*, a mould which requires merely the thiazole portion to fulfill its thiamine requirements, was used satisfactorily to assay the dialysates of thiamine-free enzyme mixtures made with thiamine incubated with either carp tissue or NaCl extracts of carp tissue. On the other hand, *Endomyces vernalis*, a yeast which requires merely the pyrimidine portion to fulfill its thiamine requirements, showed that only a small fraction of the activity of the original thiamine remained in the dialysates of thiamine-free enzyme mixtures made by incubating thiamine with NaCl *extracts* of carp tissue. When similar mixtures

made with carp *tissue*, rather than extracts, were assayed with *Endomyces* all the activity of the original thiamine was accounted for. In all cases a chemical method was used to assay for whole thiamine. Treatment with alkali of the thiamine-free reaction mixture of extract incubated with thiamine restored full activity in tests with *Endomyces*. Standing for long periods also increased the ability of these mixtures to promote the growth of the yeast. On this evidence the authors postulated a two-step reaction. The first, carried out in extracts of carp tissue, would be to split the thiamine into thiazole and a pyrimidine derivative with but slight activity for *Endomyces*. The second step, carried out by a substance present in the carp tissue but not in the extract, would serve to convert this pyrimidine derivative into 2-methyl-4-amino-5-hydroxymethylpyrimidine. This hypothesis has been investigated further by Hennessy (29) but as yet his results are not available.

In their investigations Krampitz and Woolley (35) noted that most of the thiamine-destroying potency of carp extract was lost upon dialysis against distilled water. The activity of the extract was fully restored, however, upon addition of the dialysate which in itself was inactive. The factor in the dialysate, moreover, was heat stable, whereas that in the undialyzable fraction was heat labile.

The rate of destruction of thiamine by carp extract was increased by raising the temperature and the *pH*. Temperatures over 37° C. were not tested but the temperature coefficient over the range 0 to 37° was small. Thiamine destruction proceeded at *pH* 8 almost twice as fast as at *pH* 5. At *pH* 1 the reaction went at about half the rate at *pH* 5 and the authors believe that this is due in part to the destruction of the enzyme at *pH* 1. Values above *pH* 8 were not tested. The rate of destruction of thiamine by a given amount of carp extract was determined. In one example, the cessation of thiamine destruction after the first 30 minutes, when about three-fifths of the added thiamine was split, was shown by the authors not to be due to an inhibitory action by the breakdown products. Krampitz and Woolley (35), however, give no data concerning initial *pH* or control of *pH* in any measurement of enzyme activity other than that done to find the effect of *pH*. As such, it is difficult to evaluate their results in some experiments.

*Assay of Thiamine.* At this point a few words must be said concerning the chemical determination of thiamine. In the investigations of the behavior and characteristics of thiaminase, the work of Sealock *et al.* (52), Krampitz and Woolley (35), Lieck and Ågren (37), and others to be mentioned below, the method for the determination of thiamine was a modification of that described by Melnick and Field (39). This method involves the adsorption of the vitamin on zeolite, its elution with KCl, and treatment of the eluate with diazotised *p*-aminoacetophenone (the Prebluda-McCollum (43) reaction). The resulting pigment is extracted with xylene and measured colorimetrically. In many of the studies on thiaminase, however, the zeolite adsorption was omitted. Krampitz and Woolley (35), however, adsorbed the thiamine on Super Filtrol and determined the thiamine in the adsorbates according to the method of Emmett, Peacock and Brown (15) which again is essentially based upon the Prebluda-McCollum reaction (43).

In their investigations, Lieck and Ågren (37) were rarely able to recover entirely thiamine added to fish extract the protein of which was then precipitated by trichlo-

roacetic acid; at times their loss was as much as 50 per cent. Except for their omission of the adsorption step, their procedure was that of Sealock *et al.* (52) who, although they did not report the loss of thiamine in controls, were able to assay a given extract in terms of units of thiaminase with an average error not greater than  $\pm 5$  per cent. Ågren (64) later found that, among other agents, cysteine and ascorbic acid can inhibit the formation of color in the modified Melnick-Field method. Whether this is due to the omission of the adsorption step is not certain. Sealock and Goodland (49), who also reported interference in color development by cysteine, presumably adsorbed the thiamine on to zeolite. Jacobsohn and Azevedo (32) have found that extraction of fresh gold fish (*Carassius*) with M/15 phosphate (pH 6.9) yields an extract which does not lose thiamine when the protein is precipitated; they believe that the thiamine is lost by adsorption on to the precipitated proteins rather than by a non-specific interference with the Melnick-Field color. But their recovery of thiamine may in fact be due to the use of the thiochrome procedure for the assay. Ågren (64) noted that those factors interfering with color development in the modified Melnick-Field method do not interfere in the thiochrome method. The results of Krampitz and Woolley (35) show that they were able to recover 96 per cent of the thiamine added to boiled extracts. Their direct adsorption on to Super Filtritol at pH 2-3 avoided protein precipitation with trichloroacetic acid. In all their calculations these authors imply that their recovery was 100 per cent.

It will be noted below that some investigators have used a thiochrome assay method. In view of the foregoing it would appear that this method is preferable to the use of the Melnick-Field method, especially when the latter method is modified to exclude the adsorption procedure. Sealock and Livermore (50) have suggested a manometric method for the measurement of thiaminase activity. Since the cleavage of one molecule of thiamine is accompanied by the release of a proton, these authors have measured the liberation of extra carbon dioxide in a bicarbonate buffer. Comparison with chemical determinations of thiamine lost, showed that, as developed, the method gave results that were not at all reproducible.

### Thiaminase

**Purification.** In his first efforts Ågren (61) succeeded in purifying thiaminase about ten times by isoelectric precipitation between pH 5.0 and pH 5.3. Since this method did not always give reproducible results, the following procedure was adopted (62): Finely ground fish (tench, *Tinca vulgaris*, or ide, *Leuciscus idbarus*) viscera were shaken for 1 hour at 0° C. with an equal volume of water, and then centrifuged for 1 hour at 3,000 r.p.m. in the cold. The precipitate was discarded and the centrifugate acidified to pH 5.7 and recentrifuged. The precipitate was discarded and solid ammonium sulphate was added until the centrifugate was 0.6 saturated. With Hy-flow Super Cel the precipitate was filtered off by vacuum and discarded. The clear filtrate was then completely saturated with ammonium sulphate and the resulting precipitate spun off. Dissolved in water, this precipitate gave a thiaminase preparation which showed a 10-fold increase in activity over the original water extract. Cataphoretic separation and short dialysis of the partially purified enzyme resulted in still another 10-fold increase in purity (63). The resulting solution was clear and

colorless, and was 100 times as active as the original aqueous extract of the fish viscera. The activity of most of these preparations was not stable even when the material was stored at  $-15^{\circ}$  C. Units of thiaminase activity were those employed by Sealock *et al.* (52) and were measured in terms of mg. protein nitrogen.

**Characteristics.** Inactivation of carp thiaminase by dialysis was also accomplished by Lieck and Ågren (37) in their studies on the distribution of the enzyme in various species of fishes. The activity of the enzyme prepared by extracting the fish viscera with water was estimated by the method of Sealock *et al.* (52). Lieck and Ågren (37) showed the inactivation of thiamine by carp extract was not an oxidative process since neither was oxygen uptake noted in manometric experiments nor was methylene blue reduced in experiments carried out in Thunberg tubes. Evidence for reductive inactivation was presented by Ågren (61) when he showed that the activity of dialyzed, completely inactive enzyme was restored by the addition of glutathione. The enzyme, freed from accompanying catalase by isoelectric precipitation, was then partially inactivated by hydrogen peroxide and again reactivated by glutathione but in this case the glutathione concentration was high enough to interfere with color development in the Melnick-Field method. It is doubtful whether the thiamine is reduced, since sodium hyposulphite does not interfere with the assay except when in high molar ratio to thiamine.

Whether glutathione, in activating dialyzed thiaminase, is acting as a coenzyme or reducing the enzyme to an active state has not been ascertained. The effect that glutathione exerts by activating these preparations is very likely not due to interference with color development for two reasons: 1) the concentration of glutathione is below the minimum which interferes and 2) addition of glutathione in the same concentration to an extract prepared from a fish which does not contain thiaminase does not cause that extract to show thiamine destruction under the experimental conditions. Whether glutathione, or any reducing substance, is, indeed, the true coenzyme cannot be said for certain. Ågren (61) found that extracts of fish which did not contain thiaminase could not be induced to destroy thiamine upon the addition of the dialyzable factor from extracts of fish which did contain the enzyme. No one has observed, however, whether the dialyzable factor is present in fish, extracts of which do not destroy thiamine.

Recently Sealock and Livermore (51) have restored the activity of dialyzed thiaminase with manganous chloride ( $2 \times 10^{-4}M$ ). So far, the data have not been given completely but cobaltous ions also were effective.

Bhagvat and Devi (7) used chloroform-water mixtures to extract thiaminase from carp tissue. They confirmed the heat stability of the co-factor and the comparative insensitivity of the velocity of the enzyme reaction to hydrogen ion concentration between  $pH$  4 and  $pH$  7. Extracts of the viscera destroyed 100 per cent of added thiamine (25  $\gamma$ ) in one hour at  $pH$  5.6. The thiochrome method was used for estimating thiamine.

The study of the inhibition of thiaminase is also complicated by the agents tested interfering with the development of color in the Melnick-Field method. Sealock and Goodland (48) found that carp thiaminase could be markedly inhibited by cupric, zinc and ferric ions. Cyanide, fluoride, iodoacetate, sulphydryl and sulphite

were not as effective. Such common agents as mercuric and silver ions, potassium ferricyanide, hydroxylamine, hydrogen sulphide, cysteine, iodine and sodium sulphite interfered with color development (49). Use of the thiochrome method, however, has shown that in low concentrations mercuric ion will not inhibit goldfish thiaminase (34). Neither morphine nor acetylcholine seemed to have any effect on this enzyme (33, 34).

The thiaminase activity of carp can be competitively inhibited by *o*-aminobenzyl-4-methylthiazolium chloride, which bears a structure similar to thiamine. Substitution of the amino group of this compound by a nitro group abolished its capacity to inhibit the enzyme (48). If the amino group is in the *para*- position in this compound, no inhibition occurs; *m*-aminobenzyl-4-methylthiazolium chloride increases the velocity of thiaminase action. Varying the position of the methyl group in these compounds has no effect (51). Sealock and Davis (47) showed that *meta*-substituted aromatic amines, in this case *m*-nitroaniline, combine with the pyrimidine derivative resulting from the thiamine split thereby increasing the velocity of the cleavage. This would thus serve to explain the enhancing effect of *m*-aminobenzyl-4-methylthiazolium chloride. The inhibition of thiaminase produced by  $\beta$ -aminoethyl-4-methyl-thiazolium chloride was also greatly reduced upon substitution of the amino group by a phthalimido group (48). Since oxythiamine (deaminated thiamine) also inhibits thiaminase (54) it is difficult to assess the value of the amino group in the substrate for the formation of the enzyme-substrate complex.

The thiaminase of carp spleen has been shown by Beloff and Stern (6) to inhibit yeast cocarboxylase activity, probably by actual destruction of the cocarboxylase (thiamine diphosphate).

#### DISTRIBUTION OF THIAMINASES

The investigations of the occurrence of thiaminase in different species of fishes and in other organisms has been carried out by the feeding of the fish to be tested in order to see whether Chastek paralysis could be produced, and also by the much more rapid *in vitro* method of determining the thiamine destroyed upon incubation of the vitamin with extracts or breis of the fish to be tested. In any case it is necessary that the animal tested be identified accurately. For that reason much of the early work on the distribution of thiaminase must be discounted. Common names, especially those of fishes, will often change radically from one small locality to another. Conclusive results will be reached if the whole animal or its viscera is used for the study because most of the thiaminase activity resides in the viscera (cf. table 1).

Deutsch and Hasler (12), using an *in vitro* method on whole fish or fish viscera, examined 31 species of fish caught in the Great Lakes region of the United States for the presence of thiaminase. Fifteen of these fresh-water forms were found to contain the enzyme. All 4 species of the carp family (Cyprinidae) that they examined contained the enzyme. Of 9 marine forms examined, none contained thiaminase but in the case of the cod (*Gadus morrhua*) and the haddock (*Melanogrammus aeglefinus*) the fish had been previously eviscerated. To this list of marine teleosts Yudkin (60) added 3 more, the viscera of which did not contain the enzyme. Lieck and Ågren (37) examined 30 species of fish caught in Swedish lakes, the Baltic Sea, and the Kattegat. Altogether 10 of the 30 fish examined contained the enzyme. Of the 10

species of Cyprinidae examined, 9 contained thiaminase. The gar-fish (*Belone acus*) caught in the Kattegat, thus a salt-water form, also contained thiaminase. Later, using *Phycomyces blakesleeanus* to assay thiamine, Wiken and Ågren (57) showed that the cod (*Gadus callarias*) and the asp (*Aspius aspius*) contained small amounts of thiaminase. The slight differences that were noted, however, do not appear to be significant.

Wolf (58) has described a disease of trout caused by diets containing raw fish. Examination of the central nervous system of diseased fish, moreover, showed that the histopathology of the condition was identical with that of Chastek paralysis in foxes and of Wernicke's disease in man (4). Fish with fish-diet disease exhibit loss of balance causing the fish to lie on their sides on the bottom of the container. When disturbed the fish exhibit great excitability so that they dash about violently. These side swimmers weaken, lose their appetite and eventually die. Administration of vitamin B<sub>1</sub> cures the condition. Cooked fish will not injure the trout in any manner. Wolf was able to produce this disease by feeding trout Atlantic herring (*Clupea harengus*), a salt-water form, and buckeye shiners (*Notropis atherinoides*), a fresh-water form. That herring contains thiaminase was confirmed by the work of Smith and Proutt (53) who were able to produce a thiamine deficiency in cats on a diet containing Atlantic herring. Lieck and Ågren (37), on the other hand, did not find any thiaminase in *Clupea harengus* caught in the Baltic Sea or in the Kattegat.

Clams have also been shown to contain thiaminase (Woolley, cited in Melnick, Hochberg, & Oser, 40) but the oyster has none. Species names were not given in this study but they were likely to have been *Venus mercenaria*, *Mya arenaria* and *Ostrea virginacea*. In Portugal, thiaminase has been found in the shrimp, *Penaeus carapace*, and the clam, *Tellina*, but not in the cockle, *Cardium edule*, or the crab, *Portunus marmoreus* (32). All these species are marine, but other than these slight investigations no thorough study has been made on the distribution of the enzyme among marine invertebrates.

Azevedo (5) has found no evidence for thiaminase in tortoises (*Clemmys leprosa*) or frogs (*Rana esculenta*). Actually, the experiments with *Clemmys* were equivocal.

The fresh-water mussel, *Lamellidens marginalis*, contains thiaminase with some different characteristics from that in carp. The carp enzyme possesses a pH optimum near pH 9 (52) whereas the destruction of thiamine in aqueous extracts of *Lamellidens* shows peaks of activity at two hydrogen ion concentrations, pH 3.6 and pH 6.5 (45). Evidence that, indeed, two thiaminases are present is brought forth on the fact that M/100 iodoacetic acid inhibits thiamine destruction at pH 3.6 but leaves thiaminase activity at pH 6.5 unimpaired. On dialysis the extract does not lose any activity to destroy thiamine at pH 3.6 but loses 50 per cent of the thiaminase activity at pH 6.5. Both enzymes were inhibited by M/1000 copper sulphate and activated by manganese sulphate. No additional fresh-water invertebrates have been examined.

It is notable that thiaminase occurs to a great extent in the Cyprinidae only because that is a pattern that has been at all consistent. Other than the Atlantic herring (*Clupea harengus*) and the gar-fish (*Belone acus*) no marine teleosts have been shown to contain thiaminase. Although the early reports of Green, Carlson and Evans (26) claimed that the Atlantic whiting (*Merluccius bilinearis* ?) was responsible for the development of Chastek paralysis in foxes, subsequent *in vitro*

studies (12, 60) have not demonstrated the presence of thiaminase in this form. Needless to say, no conclusions on a phylogenetic basis can be examined about the distribution of thiaminase until more data in diverse animals are available. Present evidence that two, or possibly three, thiaminases may exist also complicates the task.

#### B<sub>1</sub> AVITAMINOSIS DUE TO THIAMINASE

The natural occurrence of Chastek paralysis due to diets containing raw fish has occurred in foxes (28) and in trout (58). Putney (44) has described symptoms in dogs which he believed were those of Chastek paralysis, but the evidence is incomplete.

Experimentally, B<sub>1</sub> avitaminosis has been produced in chicks (55), cats (53), pigeons and rats (2), as well as in foxes and trout, by the simple expedient of mixing raw carp, smelt or herring in the diet. As such, thiaminase is a useful tool for the production of thiamine deficiencies (1).

About the only fish which is eaten in a semi-raw condition in western countries is the herring, which contains thiaminase. Raw or salted herring destroyed in 6 hours about 60 per cent of thiamine added to it (40). Some brands of marinated herring destroyed anywhere from 10 to 50 per cent of added thiamine. Clams have a very powerful thiaminase which will attack both added thiamine and dietary thiamine. Melnick *et al.* (40) demonstrated that a diet containing raw clams seriously reduced the availability of dietary thiamine as measured by the thiamine excretion of human subjects.

In the past, raw fish has been valuable as a source of first class protein for livestock and also for fish in hatcheries. This is especially true in winter months when other sources of protein become limited. The presence of thiaminase in many fishes, however, indicates that caution should be used before any raw fish is indiscriminately mixed with the diet. Until the possibility that the fish fed will destroy dietary thiamine is excluded the fish should be fed separately and not mixed with the rest of the ration or more preferably, it should be cooked. Canning does not entirely inactivate thiaminase, since canned carp has been shown to have caused B<sub>1</sub> avitaminosis in fish hatcheries (46).

The importance of thiaminase in human nutrition cannot, at this point, be evaluated. In countries where raw, or partly cooked, fish forms a major constituent of the diet, thiaminase may possibly be a factor contributing to B<sub>1</sub> avitaminosis.

The function of thiaminase in the organism is obscure at present. Carp blood not only contains no thiamine but will destroy any thiamine added to it (18). Unfiltered water from Connecticut lakes contains from 0.03 to 1.2  $\gamma$  of thiamine per liter of which from 7 to 39 per cent cannot be removed by filtration (31). The thiamine requirements of fish with thiaminase may be, then, ever fulfilled by the thiamine present in lake water and its plankton.

#### SUMMARY

Chastek paralysis, caused by feeding certain raw fishes to animals, is a manifestation of an acute thiamine deficiency. The Chastek-paralysis factor in raw fish is an

enzyme, called thiaminase, which splits the dietary thiamine into its pyrimidine and thiazole components. A co-factor, possibly manganous ion or glutathione, is necessary for the activation of dialyzed preparations of thiaminase. The partial purification of thiaminase is described.

Thiaminase is present in many fresh-water fishes, notably, the Cyprinidae. It also occurs in the Atlantic herring, a fresh-water mussel and several marine invertebrates. The greatest concentration of the enzyme in carp is in the viscera and gills, but none is present in the body muscle of this form. Use of thiaminase has been made in preparing thiamine-free diets for experimental animals. The significance of thiaminase in human and animal nutrition is discussed.

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